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PRINCIPAL INVESTIGATOR: PILAR BLANCAFORT, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina at Chapel Hill
Chapel Hill NC 27599-1350

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14. ABSTRACT
The transcriptional programs cooperatively required for the malignant progression of breast tumors are largely unknown. In this project we propose the isolation of Artificial Transcription Factors (TFs) for the discovery of gene panels, which cooperate during the generation of metastatic behavior. An ATF is made by linkage of a DNA-binding domain (DBD) with a transcriptional effector domain, which mediates activation or repression of endogenous genes. ATFs are typically made of arrays of Cys2-His2 zinc finger (ZF) domains. The objective of this proposal is to apply large ATF libraries to identify and regulate genes that cooperate during the process induction of breast cancer cell invasion and progression. We have delivered ATF libraries into non-invasive breast cancer cell lines. We have selected ATFs able to induce or enhance breast cell invasion. The ATF-selections were performed in vitro using matrigel invasion assays (Boyden chambers). ATFs modulating cell invasion were profiled using DNA microarrays to determine genes differentially regulated by the ATF that are responsible for the phenotype change.

15. SUBJECT TERMS
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Blancafort, Pilar – Annual Progress report, year 2

INTRODUCTION

Metastatic spread, and not primary tumor burden, is the leading cause of breast cancer deaths. The development of metastatic behavior during breast cancer progression is a dynamic process thought to require the concerted action of multiple genes (1). The transcriptional programs cooperatively required for the malignant progression of breast tumors are largely unknown. In addition, novel therapeutic strategies should be able to target multiple targets dysregulated during disease progression. In this project we propose the isolation of Artificial Transcription Factors (TFs) for the discovery of gene panels, which cooperate during the generation of metastatic behavior. An ATF is made by linkage of a DNA-binding domain (DBD) with a transcriptional effector domain, which mediates activation or repression of endogenous genes (2-3). ATFs are typically made of arrays of Cys2-His2 zinc finger (ZF) domains (4-6). Importantly, ATFs these ZF domains can be linked to both activator and repressors of transcription, facilitating both up- and down-regulation of tumor cell phenotypes (2-3). We have developed a novel genome-wide approach for the functional identification and regulation of genes involved tumor progression. We have generated libraries of ATFs by recombination of large repertoires of sequence-specific zinc finger (ZF) domains (7-10). ZF domains were linked to an activator (VP64) or repressor (SKD) of transcription (2-3). When delivered into tumor cell populations, ATF libraries have the potential to activate or repress virtually any gene. The objective of this proposal is to apply ATF libraries to identify and regulate genes that cooperate during the process induction of breast cancer cell invasion and progression. We have delivered ATF libraries into non-invasive breast cancer cell lines. We have selected ATFs able to induce or enhance breast cell invasion. The ATF-selections were performed in vitro using matrigel invasion assays. The ATFs modulating cell invasion were profiled using DNA microarrays to determine genes differentially regulated by the ATF that are responsible for the phenotype change. We expect this proposal will lead to the functional identification of novel markers of breast cancer disease progression that could be used as early predictors of malignant behavior. In the future, these ATFs could be used as master genetic switches to modulate malignant behavior in *in vivo* models of breast cancer.

BODY

In this section we will describe the main results and conclusions for the tasks outlined in the statement of work for year 2 (months 12-24) of this proposal.

List of Tasks and expected outcomes:

Task 2. To determine the group of genes differentially regulated by the ATF that are responsible for enhancement of cell invasion (months 12-24)

a) *To obtain a list of genes differentially regulated (up- and down-regulated) by the ATFs using DNA microarrays (months 12-16).*

a1. Infect host cells with ATF DNA from Aim 1, pellet cells, extract RNA: month 12-13.

a2. Microarray hybridization and data analysis (UNC Microarray core facility, months 13-16)

Final product months 12-16: we expect to have a group of target genes differentially regulated by the ATFs.

Obtained Results and Discussion:

Previous work and follow-up work from year 1.

During year 1 we have successfully selected ATFs inducing invasive behavior in the non-invasive MCF-7 cell line with 3ZF retroviral libraries linked to either the VP64 activator domain and the SKD repressor domain. The retroviral vectors used for the transductions were the pMX-IRES-3ZF-library-VP64 and pMX-IRES-3ZF-library-SKD. The GFP marker expressed in the retroviral vector allowed the tracking of the ATF-transduced cells by flow cytometry. In year 1 we have mainly focused our analysis on the ER+ non-invasive MCF-7 cell line. These cells were used for the selection experiments described in **Figure 1**. We have recently generated in the lab a MCF-7 cell line expressing a luciferase marker (MCF-7-luc), which will allow us to follow invasive behavior in real time in a mouse model using Bioluminiscence Imaging, BLI (year 3, task 3 of our statement of work)

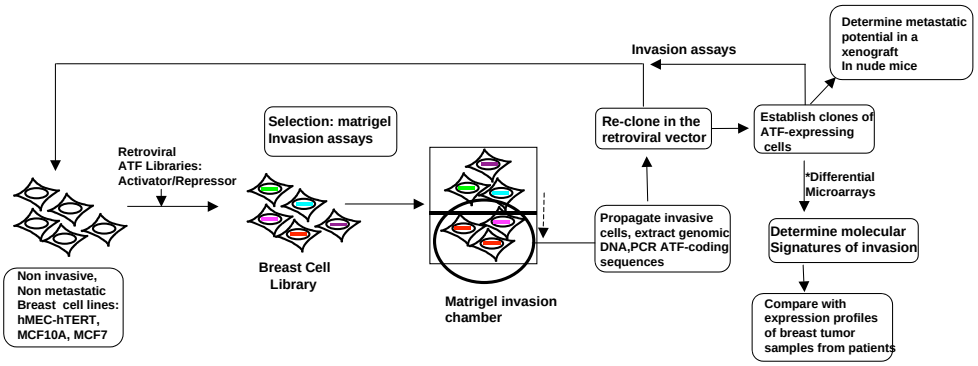

Figure. 1. Strategy to select ATFs able to regulate *in vitro* breast cancer cell invasion using matrigel-coated transwells. Cells are transduced with ATF libraries linked to activator or repressor domains. Transduced cells are genetically different and comprise a breast cell library. In the library the ATFs have the capability to up- or down-regulate any gene in the human genome. Thus, the transduced cells display phenotypic plasticity. In order to select for ATFs up-regulating cell invasion we used boyden chambers. Transduced cells are loaded in top compartment of the transwell in serum-free media and stimulated to invade by adding 0.1-2% serum in the bottom well. Non-invading cells will be removed from the upper well. Cells able to pass through the matrigel matrix are recovered and propagated. ATFs are re-cloned in the retroviral vector for next rounds of selections, as described above. Individual ATF expressing cells were isolated for invasion assays, gene expression analysis (year 2) and mouse-based studies (year 3).

In the previous report, we have described the selection of ATFs from 3ZF pools in the MCF-7 cell line. We have performed three rounds of selection for both activator, and repressor libraries. We have additionally isolated individual ATFs able to induce cell invasion in the MCF-7 cell line (Figure 2). We have sequenced seven clones to verify the variability of the ZF pools. Based on the sequences of the ZF helices we have determined the potential ATF-binding sites based on the predicted specificity of the ZF lexicons. In Figure 3, we have shown the potential binding site for each individual activator clone. We have chosen Clone 3, Clone 15 and Clone 20 for further experiments based on their potential to induce an invasive phenotype in MCF-7 cells.

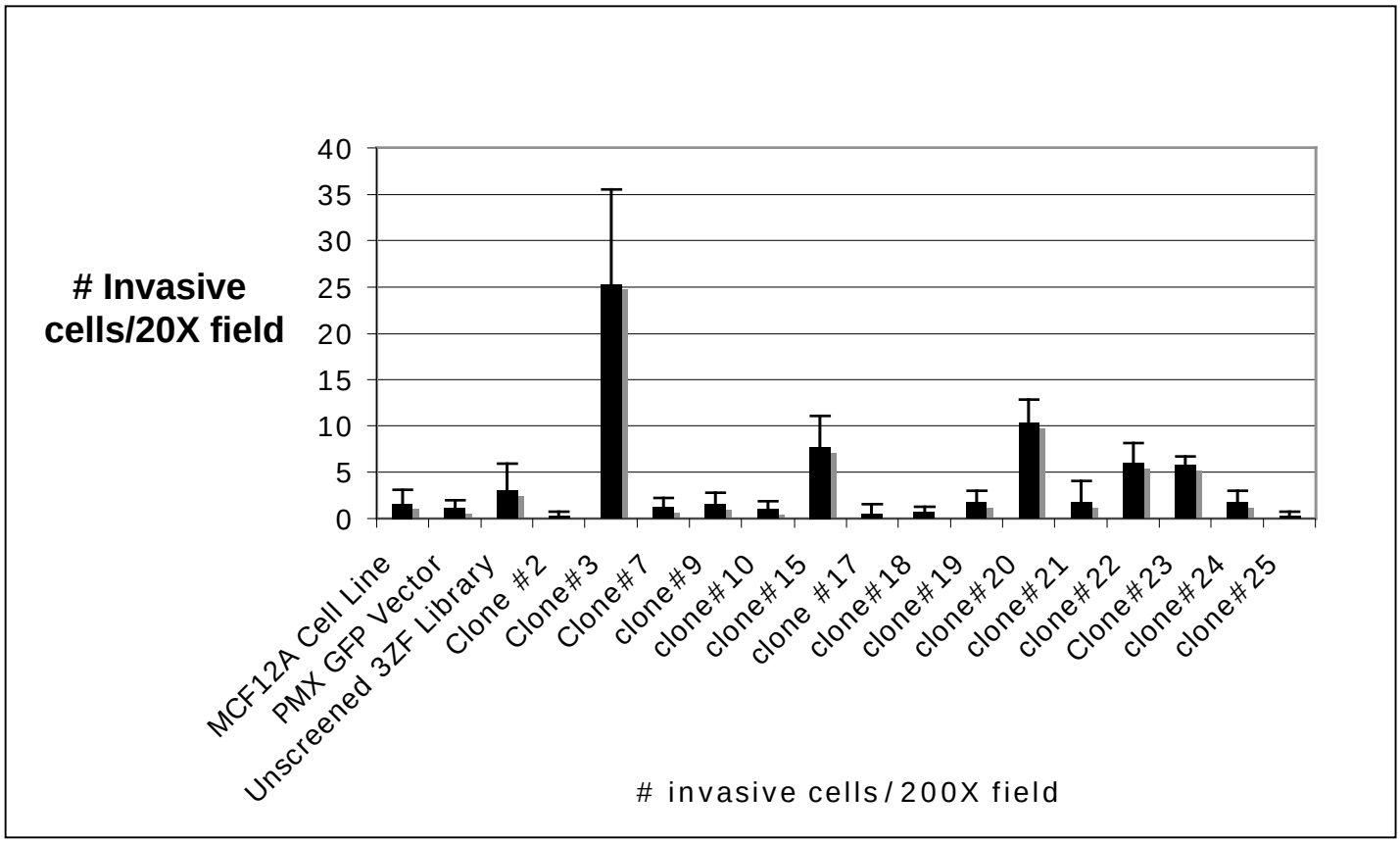

Figure 2. ATFs promote cell invasion in the MCF-7 breast cancer cell line. MCF-7 cells were transduced with individual pMXactivator 3ZF clones selected by matrigel invasion assays. Transduced cells were collected and processed by matrigel invasion assays, in 24-well format. Cells that passed through the matrigel were fixed, stained, and counted under an inverted tissue culture microscope. Three wells per clone were counted (four fields per well). The average of 12 fields and standard deviations are indicated.

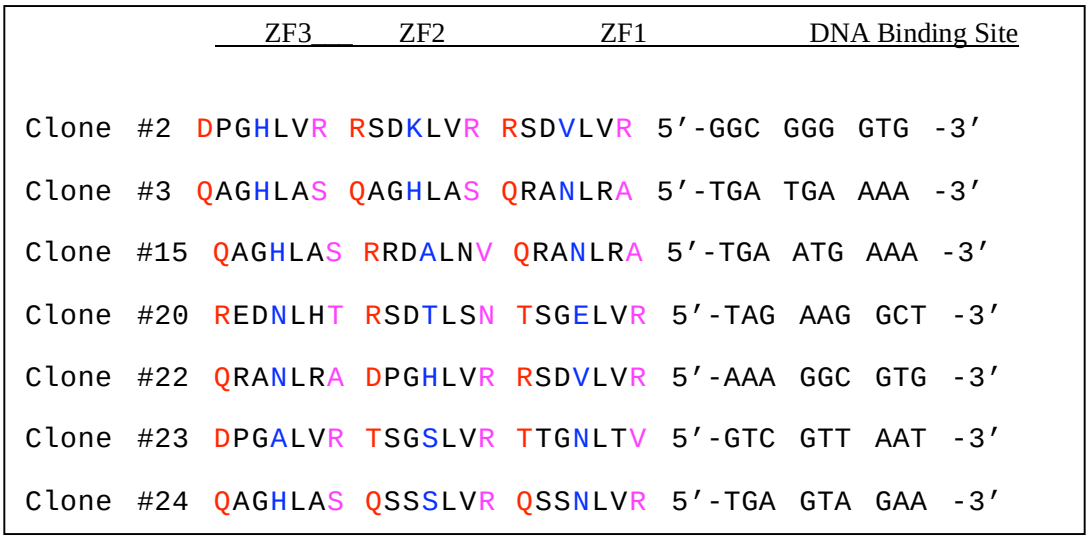

Figure 3. Predicted DNA binding sites for 3ZF activator clones. Individual 3ZF activator clones that exhibited enhanced invasive behavior were sequenced. The ZF DNA binding sites were obtained determined according to the predicted specificity of the individual ZF lexicons. Aminoacids shown in red, blue and pink represent the ZF α-helical positions –1, +3 and +6, which contact nucleotide positions 3', middle, and 5', respectively.

In this second year, we have additionally sub-cloned the ZF inserts (clones 3, 15, 20, Figure 2) from the pMX-3ZF-VP64 (activator) selected constructs into the pMX-3ZF-SKD vector, which allows the expression of the ZFs linked to a repressor effector domain. The resulting ZF transcriptional repressors were transduced into the metastatic MDA-MB-231 breast cancer cell line. The objective of these experiments was to determine if the repressor constructs were able to knock down cell invasion in the highly invasive MDA-MB-231 cells. As shown in Figure 4, these clones were able to reduce cell invasion by 90, 50 and 35%, respectively. Invasion data was normalized to cells transduced with a control vector (empty vector).

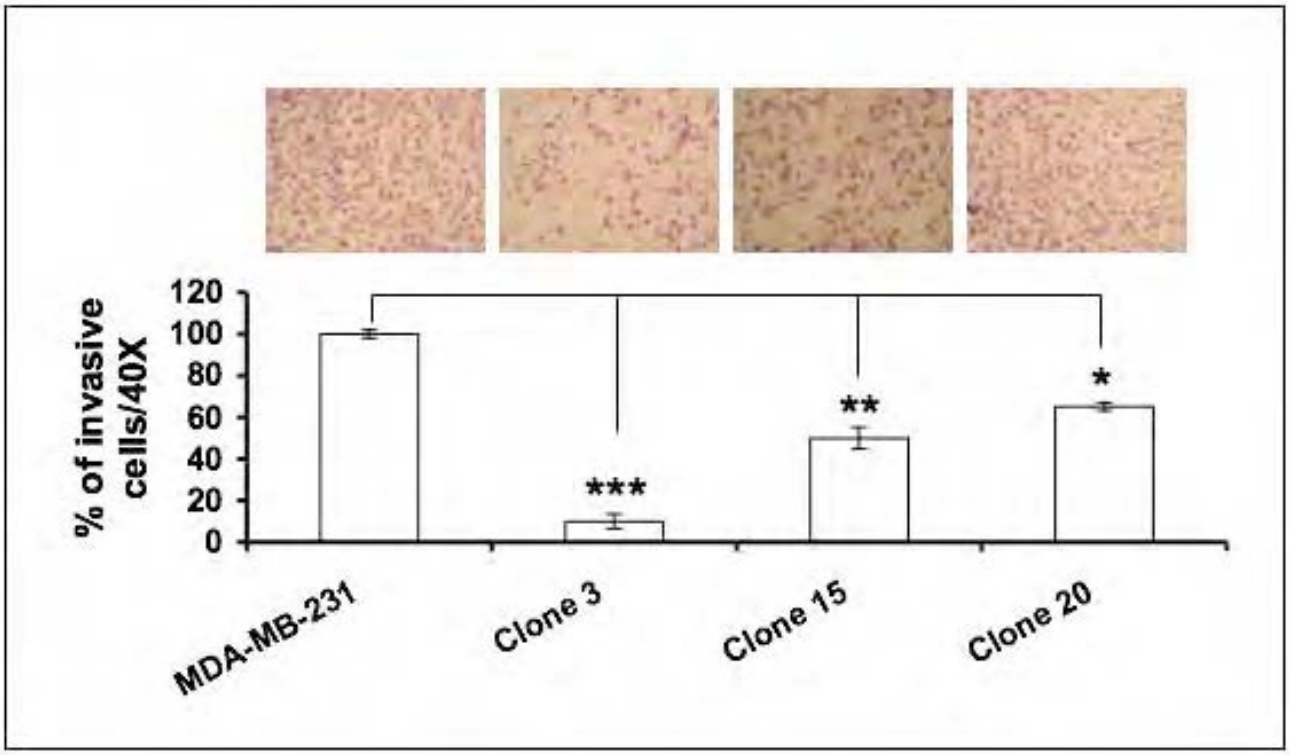

Figure 4. Individual 3ZF-SKD clones knock-down cell invasion when transduced in MDA-MB-231 breast cancer cell line. MDA-MB-231 cells transduced with the repressor vectors pMX-3ZF-SKD-Clone-3, Clone-15 and Clone-20 and control cells (transduced with an empty retroviral vector) were assayed for invasion using 24-well format Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA) 120 h post-transduction. Cells that passed through the matrigel were fixed, stained, and counted under an inverted tissue culture microscope. Invasion data was normalized to control cells. Data represent an average of three different experiments, 2 wells per clone were tested and four fields per well were counted (*P=0.05, **P=0.01, ***P=0.001, as determined by Student's t-test).

We next focused subsequent initial expression studies on Clone-3 since it was able to strongly down-regulate cell invasion in vitro. We investigated possible genes regulated by the activator ATF clone 3 by microarray analysis. We transduced MCF-7 (non-invasive) cells with a control vector (empty retroviral vector) and with the pMX-3ZF-VP64-Clone-3 (activator). We used the oligonucleotide microarrays created in the UNC-Chapel Genomics Core Facility representing 18,861 human genes (http://genomicscore.unc.edu/). Both clone pMX-3ZF-VP64-Clone-3 and control cells were processed with three independent biological samples and with three different arrays.

MCF-7 cells ($5x10^6$) were transduced with pMX-3ZF-VP64-Clone-3 and Control retroviral construct. Cell lysates from transduced cells (6 hr post-transduction) were homogenized by passing over a QIAshredder spin column (Qiagen) and total RNA was isolated from the cell pellet using the RNeasy Mini Kit (Qiagen). The synthesis of labeled cDNA and hybridizations were performed in the UNC-Chapel Hill genome Core. We performed 3 independent microarray experiments for Clone 3 and Control cells. Microarray data was imported into GeneSpring 6.1 (Silicon Genetics) and Excel (Microsoft). Genes that are significantly induced or repressed were identified using the Significance Analysis of Microarrays (SAM) package Add-In for Micrososft Exel at

the Bioinformatics core facility at UNC. We compared the expression profiles of the pMX-3ZF-VP64-Clone-3 transduced cells with control cells to determine the genes differentially regulated by the ATF-Clone-3. Table 1 shows the genes differentially regulated by ATF-Clone-3 relative to control cells (empty-virus transduced cells). These arrays revealed a robust collection of 7 targets being differentially regulated by this ATF. Most of these targets are important genes known to control cell cycle and control of cell morphology. We performed real-time quantification using Taqman assays (Applied Biosystems) to verify the up-regulation of six of these targets. We saw a robust up-regulation for all genes, but especially for Par-4/PAWR witch is a pro-apoptotic protein that is decreased in cancer cells (Figure 5).

Table 1. Genes differentially regulated

Gene ID	Gene name	Function	Fold regulation
NM_001261	Cyclin-dependent kinase 9 (CDK9)	Positive transcription elongation factor b, which facilitates the transition from abortive to production elongation by phosphorylating the ctd (c-terminal domain) of the large subunit of RNAp	2.9
NM_044472	Cell division cycle 42 (CDC42)	Regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression	1.6
NM_014255	Transmembrane protein 4 (TMEM4)	Positive regulator of neurite outgrowth by stabilizing myosin regulatory light chain	0.6
NM_002583	PRKC, apoptosis, WT1, regulator	Transcriptional activator/repressor	10.6
NM_001964	Early growth response protein 1 (Egr-1)	Transcription factor involved in early response signaling	4
NM_005348	Heat shock 90-kDa protein 1-α	Stress-related molecular chaperone	3.36
NM_005347	Heat shock 70-kDa protein 5	Stress-related molecular chaperone	2.78

We have processed by microarray analyses the the pMX-3ZF-VP64-Clone-15 and pMX-3ZF-VP64 Clone-20 transduce MCF-7 cells. presently they are being analyzed at the bioinformatics core facility. We are also processing the pMX-3ZF-VP64-Clone-3, Clone-15 and Clone-20 in a time-course manner (4, 8, 12 hrs post-transduction) by DNA-microarrays. The purpose of this time-course experiments is to evaluate also indirect targets of the ATF or downstream genes. We also, plan to cluster the up-, down-regulated genes with available transcriptional profiles of metastatic breast cancer specimens that are available at UNC from our collaborator Dr.CM Perou. In collaboration with Dr. Brenda Temple in the bioinformatics core we will study if ATF-binding sites (Figure 3, Clone 3, Clone-15 and Clone-20) are present in the promoter of the up-regulated genes (some shown in Table 1).

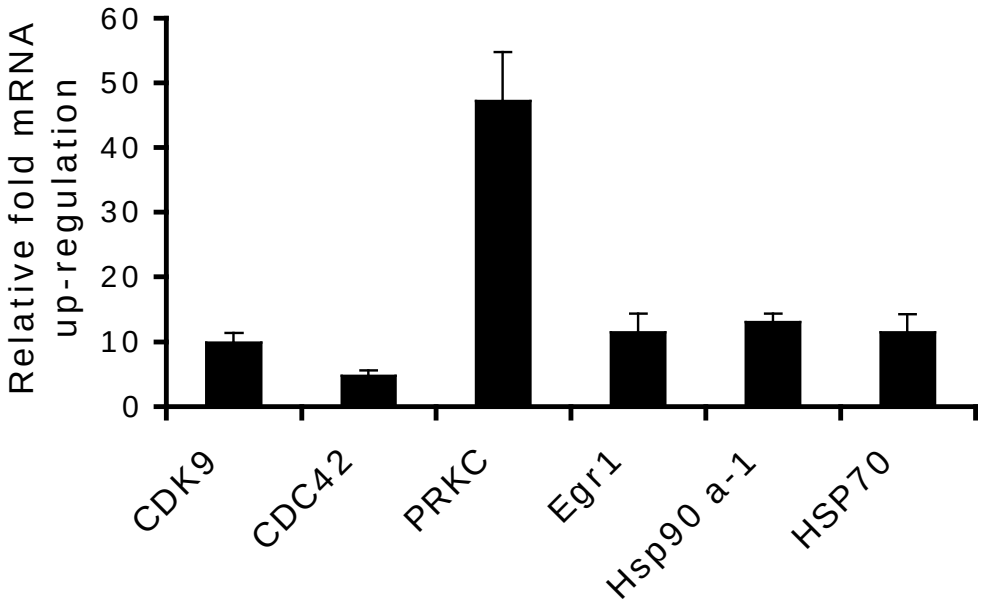

Figure 5. ATF#3 up-regulated expression of CDK9, Cdc42, PRKC, Egr1, Hsp90 and Hsp70. Real-time quantification of microarray differentially up-regulated genes. Cells were transduced with a control retroviral vector and ATF#3. After 6 h post-transduction, cells were collected, RNA extracted and processed by real-time PCR. The relative fold up-regulation was calculated using cells traduced with a control vector and the $2^{\Delta\Delta Ct}$ method and GAPDH as a normalized control. Real-time quantification is an average of three independent experiments.

KEY RESEARCH ACCOMPLISHMENTS

-Selection of pools of ATF activators able to induce invasive behavior in vitro using the MCF-7 host cell line for the selections

-Isolation of single ATF activators with capability to up-regulate and down regulate cell invasion in the MCF-7

-Isolation and characterization of ATF-REPRESSORS able to down-regulate invasive behavior in the metastatic MDA-MB-231 breast cancer cell line.

-The identification of potential ATF-targets for ATFclone#3 (linked to an activator domain). These targets could mediate regulation of cell invasion and metastases.

REPORTABLE OUTCOMES

ABSTRACTS AND MANUSCRIPTS:

* Beltran, A., Liu, Y., Parikh, S., Brenda, T., and Blancafort, P. Interrogating genomes with combinatorial transcription factor libraries: asking zinc finger questions. *Assay Drug Dev Technol*. 2006; 4:317-331

- Beltran, A., Parikh, S., Liu, Y., Cuevas, BD, Johnson GL, Fustcher, BW and Blancafort, P. Reactivation of a dormant tumor suppressor by designed transcription factors. *Oncogene*. 2007;26:2791-8.

- Beltran, A., Sun, X., Lizardi, PM and Blancafort, P. Reprogramming epigenetic silencing: Artificial Transcription Factors synergize with chromatin remodeling drugs to re-activate the tumor suppressor *maspin. Mol Cancer Ther*. 2008; *In Press*

- Blancafort P, Beltran AS. Rational Design, Selection and Specificity of Artificial Transcription Factors (ATFs): The Influence of Chromatin in Target Gene Regulation. *Comb Chem High Throughput Screen.* 2008;11:146-58.

- Beltran, AS and Blancafort, P. Reprogramming epigenetic silencing with Artificial Transcription factors. April 15th, 2008. AACR, experimental and molecular cancer therapeutics minisymposium. San Diego, CA.

AWARDS

-V-Foundation award for breast cancer research to Pilar Blancafort, May 2005

-SPORE breast cancer award UNC-Chapel Hill

-UCRF research innovation award, Lineberger Comprehensive Cancer center, The University of North Carolina at Chapel Hill

-Golfer against Cancer research award, UNC-Chapel Hill

CELL LINES AND CLONES

-Retrovirally-transduced MCF-7 cells able to invade in vitro

-ATF-sequences and clones able to up-regulate cell invasion in the MCF-7 cell line

-Retrovirally transduced MDA-MB-231 cells able to reduce cell invasion

-Novel potential biomarkers of invasion/metastatic ability in the MDA-MB-231 breast cancer cell line.

-3ZF clones 3, 15 and 20 able to modulate cell invasion in the MCF-7 and MDA-MB-231 breast cancer cell line

CONCLUSION

In this report we have shown that we were able to isolate ATFs made of 3ZF domains linked to the VP64 activator domain up-regulating and down-regulating cell invasion in the non-invasive breast cancer cell line MCF-7 and in the invasive cell line MDA-MB-231. Several clones of ATFs were isolated and their sequences were characterized. The most powerful ATFs was processed by DNA-microarray in order to understand which group of target genes are involved in activation of invasive programs. Multiple ATF targets were identify with potential effects on cell cycle control, differentiation and cell morphology.

"So What Section"
The molecular programs and genetic cascades responsible for the development of metastatic behavior remains largely unknown. Thus, there is a need to dissect genes that cooperate for the development of metastasis. The ATFs isolated in our work can provide clues about the target genes that generate metastatic behavior. In addition, the advantage of ATFs is that they can be used in the future as direct regulators of these genes. An ATF can be linked to different effector domains, which can repress or silence gene expression. Thus, we hope that the future modification of these ATFs could interfere with gene expression and function in metastatic cell lines. ATFs made of 3 ZF domains directed against the VEGF gene are presently in clinical trials. Thus, we hope that the result of our work could lead to the future development of anti-metastatic reagents.

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(7) Blancafort, P., Magnenat, L. and Barbas, C.F.III. (2003). Scanning the human genome with combinatorial transcription factor libraries. *Nat. Biotech*. 21, 269-274.

(8) Lund, C.V., Blancafort, P., and Barbas, C.F.III. (2004). Promoter targeted phage display selections with preassembled synthetic zinc finger libraries for endogenous gene regulation. *J. Mol. Biol.* 340, 599-613.

(9) Magnenat, L., Blancafort, P., and Barbas, C.F.III. (2004). *in vivo* selection of combinatorial libraries and designed affinity maturation of polydactyl zinc finger transcription factors for ICAM-1 provides new insights into gene regulation. *J. Mol. Biol.* 341, 635-49.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Blancafort, Pilar	Assistant Professor of Pharmacology
eRA COMMONS USER NAME PILAR_BLANCAFORT	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Universitat de Barcelona	B.Sc.	1988-1993	Molecular Biology
Université de Montréal	Ph.D.	1995-1999	Biochemistry
The Scripps Research Institute	Res. Assoc	1999-2003	Biochemistry
The Scripps Research Institute	Senior Res. Assoc.	2003-2004	Biochemistry
The University of North Carolina at Chapel Hill	Ass. Prof.	2005-present	Pharmacology

A. Positions and Honors

Positions and Employment

1988-1993 -B.Sc., Molecular Biology and Biochemistry, Universitat de Barcelona (Spain)
Personal Project and last year of B.Sc (1992-1993)
Universite Libre de Bruxelles (Belgium) with Dr. Alain Ghysen

1994-1995 - M.Sc. Program, Biochemistry, Université de Montréal (Canada)

1995-1999 - Ph.D. Program, Biochemistry, Université de Montréal with Robert Cedergren

1999-2003 - Research Associate in Dr. Carlos Barbas, III laboratory, Department of Molecular Biology, The Scripps Research Institute

2003-2004 -Senior Research Associate in Dr. Carlos Barbas III laboratory, Department of Molecular Biology, The Scripps Research Institute

2005 -Assistant Professor, University of North Carolina at Chapel Hill, Department of Pharmacology
Member, Lineberger Comprehensive Cancer Center.

2007 Member, American Association for cancer research, AACR

Teaching Experience

1994-1997 -Teaching assistant, biochemistry undergraduate laboratory, Faculté des Arts et des Sciences, Université de Montréal

2002 -Supervised summer High school teacher Paul Messier, The Scripps Research Institute

2003 -Supervised College student Leanna Lagpacan, The Scripps Research Institute

2004 -Supervised Ph.D student Sharon Bergquist, The Scripps Research Institute

2005, 2006 -I taught the Pharmacology graduate studies courses: Techniques in gene manipulation, seminars in Pharmacology, and nuclear receptors

Scholarships and awards

1992-1993 -EEC ERASMUS fellowship to finish last year of B.Sc. in ULB (Université libre de Bruxelles Belgium), Fellowship from Fondation Jean Branchet (ULB, Université Libre de Bruxelles) to support foreign students at the ULB

1998 -Winner, Simon-Pierre Noel Price Department of Biochemistry, University of Montreal.

1994-1999 -FES (Faculté des Études Supérieures) fellowship to support foreign students in the University of Montreal

1999-2004 -Research supported by Novartis Pharma fellowship (Oncology)
2005 -University Research Council Award, UNC-CH
-Lung Cancer Discovery Award
-GI SPORE Reseach Award
-V Foundation Award in cancer research (breast cancer fellowship in honor of Julie Stewart)
2006 -Department of Defense Idea award (breast cancer).
2007 - Carolina Center of Cancer Nanotechnology Excellence (C-CCNE) pilot grant award
-Golfers Against Cancer Award

Patents: Blancafort, P. and Barbas III, C.F. "Zinc finger libraries". International Patent Application Serial No PCT/US03/03705 (2001).

B. Peer Reviewed Publications

(* most significant to proposed work)

Blancafort, P., Ferbeyre, G., Sariol, C., and Cedergren, R. Pol I-driven integrative expression vectors for yeast. *Journal of Biotechnology* 1997;56:41-7

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Blancafort, P and Beltran, A. Rational design, selection and specificity of Artificial Transcription Factors (ATFs): the influence of chromatin in target gene regulation. Review. Special issue of CCHTS, *In Press*.

Published abstracts:

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The major goals of this project are the selection of ATFs that induce cell invasion in breast epithelial cells. We want to study if ATF, delivered in highly invasive breast cells, can modulate metastasis in vivo using a mouse model.

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The major goals of this project is to design, construct and characterize artificial transcription factors made of 6 zinc finger domains that are able to re-activate the mammary serine protease inhibitor (maspin) gene in metastatic breast cancer cells

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Yale University/NIH
Probes for Detection of DNA Accessibilty in Chromatin
The Blancafort Laboratory will be responsible for the construction of 6 zinc finger DNA-binding domains, the sub-cloning of those domains in several bacterial and mammalian-expression vectors and for the characterization of those domains both in vitro and in vivo.

Rational Design, Selection and Specificity of Artificial Transcription Factors (ATFs): The Influence of Chromatin in Target Gene Regulation

Pilar Blancafort* and Adriana S. Beltran

Department of Pharmacology and the Lineberger Comprehensive Cancer Center, the University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365, USA

Abstract: Artificial Transcription Factors (ATFs) are engineered DNA-binding proteins designed to bind specific sequences of DNA. ATFs made of Zinc Finger (ZF) domains have been developed to regulate specific genes and phenotypes both in cells and whole organisms. Recently, an emerging application of engineered DNA-binding domains include the specific editing of the genome, the ability to specifically cut, recombine, modify DNA and image protein-nucleic acid interactions in living cells. In this review we will summarize the techniques used for the rational design, screening and functional selection of ZF proteins in mammalian cell systems and their applications in areas of biotechnology, functional genomics and molecular therapeutics. The *in vivo* specificity of the engineered ATFs will be discussed, with particular emphasis on epigenetic modifications influencing ATF-DNA interactions.

INTRODUCTION

In eukaryotes, the majority of chromosomal DNA carries non-coding functions. Complex genomes had to find ways not only to encapsulate information for decoding gene to protein, but also to ensure the proper spatio-temporal sequence of gene expression. Transcription Factors (TFs) are proteins which bind DNA and orchestrate which genes are going to be expressed in any given cell type and at any given time. The number and diversity of TFs appears to be higher in more complex genomes, indicating that TFs play an essential role in generating phenotypic plasticity. In segmented organisms, sophisticated TF-regulatory networks ensure the functional specification of each body part. It is not surprising that mutations, re-arrangements or improper expression of TFs often result in severe changes in phenotypes and in fatal diseases such as cancer. It is probable that the TF "pool" or TF-repertoire, the collection of TFs available for genomes to control gene expression, could contribute to phenotypic plasticity during development, evolution of new organism's body plans and also during neoplastic transformation (Fig. **1**).

The major class of TFs comprises the Cys_2-His_2 zinc finger (ZF) DNA-Binding Domain (DBD). There are 900 ZF proteins encoded in the human genome, representing approximately 2% of its coding capacity [1-4]. Distinctive features of ZF-containing proteins revealed by structural analyses include their DNA-sequence selectivity and multi-modularity. Arrays of ZF domains "travel" around the DNA major groove and read structural information by making specific H-bonds with the DNA nucleotides. The side chains of the ZFs recognize one or more strands of the double stranded DNA in an antiparallel recognition mode, similarly to a triple-helix configuration. The discovery of specific "rules" of recognition between the ZF's amino acid side chains and the DNA led to the emerging field of engineering Artificial Transcription Factors (ATFs). The goal of transcription factor bioengineering is to change the specificity of transcription factors by modifying the amino acid residues that are responsible for direct contact with the DNA. The resulting ATF can then be used to bind specific sequences in a complex genome, to perturb gene expression at a single locus level or by altering the entire transcriptional network, to modify DNA, and to image DNA-protein interactions in a given cell.

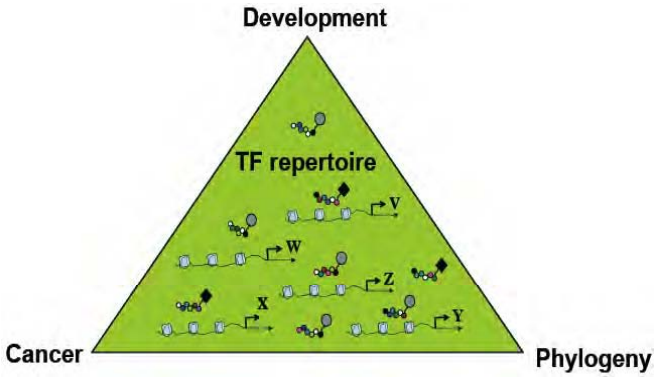

Fig. (1). Genomes control gene expression using a transcription factor repertoire, which leads to phenotypic plasticity during development, evolution of new organisms or neoplastic transformation.

Many excellent reviews in the topic of ATFs have been published in the last five years [5-13]. In this paper we will review the methodologies used to isolate designed DNA-binding domains with special emphasis on high-throughput cell-based screening assays for the isolation of ATFs with functional activity *in vivo*. When delivered in mammalian cells or even in whole-organisms, ATFs can be selected to induce complex phenotypes and reprogram cells. Related to this point, we will also discuss the importance of chromatin structure or "chromatin context" in defining the specificity of an ATF for a given target gene. With the emergence of the novel field of epigenetics, a new paradigm addressing the "*in vivo*" specificity of ATFs is arising. In a context of a living cell, specificity might not simply be dictated by a "3D-recognition code" defining the stereochemical interaction between amino

*Address correspondence to this author at the Department of Pharmacology and the Lineberger Comprehensive Cancer Center, the University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365, USA;
E-mail: pilar_blancafort@med.unc.edu

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acid residues and the DNA base pairs. Instead, it is becoming clear that a major component of specificity is defined by the chromatin structure, which influences the accessibility of an ATF-binding site. Since chromatin topology is a rather dynamic process, with a reversible nature, we now have to think about ATF-DNA interactions as a rather plastic event, subjected to "chromatin rules". The language of epigenetics, the combination of specific modifications of the chromatin (or "chromatin marks") is still far from being comprehended and intensive research is now exploring the underlying grammar. The same chromatin rules most likely will dictate the specificity of binding and regulation by ATFs.

ENGINEERING ARTIFICIAL TRANSCRIPTION FACTORS

Typically, designer transcription factors or Artificial Transcription Factors are modular proteins composed of an engineered DNA-binding-domain (DBD) which is intended to recognize a specific "targeted" genomic sequence, and an effector domain (ED) which positively or negatively regulates transcription. A variety of enzymatic functions can be coupled to the engineered DNA binding domain, enabling modifications in the chromatin in a sequence-specific manner or "genome editing". Of particular interest are the site-specific nuclease and recombinase functions, which could mediate the replacement of mutated alleles of a gene for gene therapy purposes.

ENGINEERING NOVEL DNA-BINDING DOMAINS USING THE CYS2-HIS2 ZINC FINGER (ZF) SCAFFOLD

As described above, the archetype of protein architecture used to evolve novel DNA-binding specificities is the Cys_2-His_2 Zinc Finger (ZF) domain [14]. Each ZF is a compact 30-amino acid domain where a recognition α-helix is packed against two antiparallel β-strands *via* the coordination of a zinc ion with two cysteine and two histidine residues [15]. The recognition α-helix recognizes three or four base pairs (bps) of DNA by making specific H-bonds in the major groove (Fig. **2**). The framework used for engineering ZF proteins is based on the Zif268 mouse 3ZF transcription factor, which mainly recognizes one strand (also named G-rich strand) of the double stranded DNA [1-2]. However, other ZF proteins recognize both strands of DNA and the exact mode of recognition depends on the way that the α-helix is positioned or docked in the DNA major groove. Although the ZF is an archetypal domain used for engineering novel DNA-binding proteins, the same domain has been shown to bind specifically to RNA and also DNA-RNA hybrids [16-17]. Since the major groove of the double stranded RNA is narrower and deeper (a conformation named "A form") than the corresponding major groove of dsDNA ("B form") most ZFs binding RNA such as TFIIIA recognize RNA-conformations that locally open the major groove [18]. These RNA structures comprise non-canonical Watson-Crick base pairs or nucleotides adjacent to loops, where the major groove is locally more accessible for docking the ZF recognition helix [17].

Albeit the small size of the domain, ZFs have essential features that have been exploited for engineering purposes. First, the recognition helix is capable of sequence discrimination and most ZFs are able to selectively bind distinct DNA-binding sites in a sequence-specific manner. Second, most ZF proteins are multimodular ("polydactyl"), comprising two or more ZF building blocks. Importantly, each ZF recognizes their base pairs in a quasi-independent manner. This has allowed investigators to modify the DNA-binding specificity of particular domains with minimal structural perturbations on neighboring domains. On the other hand, these experiments lead to the development of a "chemical recognition code" or the establishment of "rules of interactions" between a "generic" ZF helix and a DNA-triplet. In the Zif268-DNA complex there is an inter-domain contact between the ZF units and the DNA triplets established by position +2 of the α-helix, which interacts with C or A in the complementary strand ("C-rich") of the neighboring triplet recognizing the previous ZF domain (Fig. **2**). In addition, it is important to note that the specific sequence of nucleotides in ZF binding sites or in the neighboring nucleotides might create a "context dependence" and can potentially modify the DNA-helical parameters or trigger conformational changes in the DNA upon ZF-binding, as described for T/A rich regions binding the ZF protein Tramtrack (TTK) [19]. In any scenario, the recognition between the ZF and the nucleic acid needs to be understood in terms of 3-D structure of both the nucleic acid and the protein. Thus, the nature of the recognition code between the ZF and the nucleic acid is "stereo-chemical", with the protein "reading" structural information provided by the nucleic acid. As more structural information between ZF-DNA contacts became available it was clear that the nature of the recognition between a given DNA triplet and the ZF-α-helices was "degenerated". Many possible α-helices are able to bind the same triplet, but with different degrees of specificity and selectivity. Certain ZFs were even found not to have sequence discrimination at all [20-21]. Third, the 3-D structure of polydactyl ZF proteins provides a continuum of H-bonding interactions between the ZF's α-helices and the DNA, similar to the way that a DNA oligonucleotide would pair with one of the strands of the double helix. The N-terminal residues of each α-helix provide this continuum of H-bonding interactions. As shown in Fig. **2a**, the C-terminus of the ZF's α-helix is too far from the DNA to generate H-bonding interactions. The sequence of the four amino acid linkers, which is highly conserved between the Cys2-His2 class, guarantee the binding of the ZF's helices to contiguous DNA triplets. Thus, the linkers do not play a direct role on DNA-binding but rather allow the optimal docking of the helices in the DNA major groove. The engineering of optimized 8-amino acid linkers between 3ZF moieties had resulted in ZF- DNA recognition with improved dissociation constants in the femtomolar range [22-23]. Wang & Pabo developed a scheme that allows head-to-head dimerization of zinc fingers, which were obtained by adding random 15-amino-acid extensions to the N terminus of a two-finger region, then selecting and optimizing peptide sequences that allowed protein dimeric binding [20,24]. Recently, the design of a poly-Arginine linker between two 3ZF moieties has mediated the recognition of discontinuous target sites [25].

IN VITRO AND *IN VIVO* STRATEGIES FOR THE SELECTION ZF DOMAINS RECOGNIZING DNA TRIPLETS

The mouse transcription factor Zif268 was used as framework for the construction and isolation of novel ZF

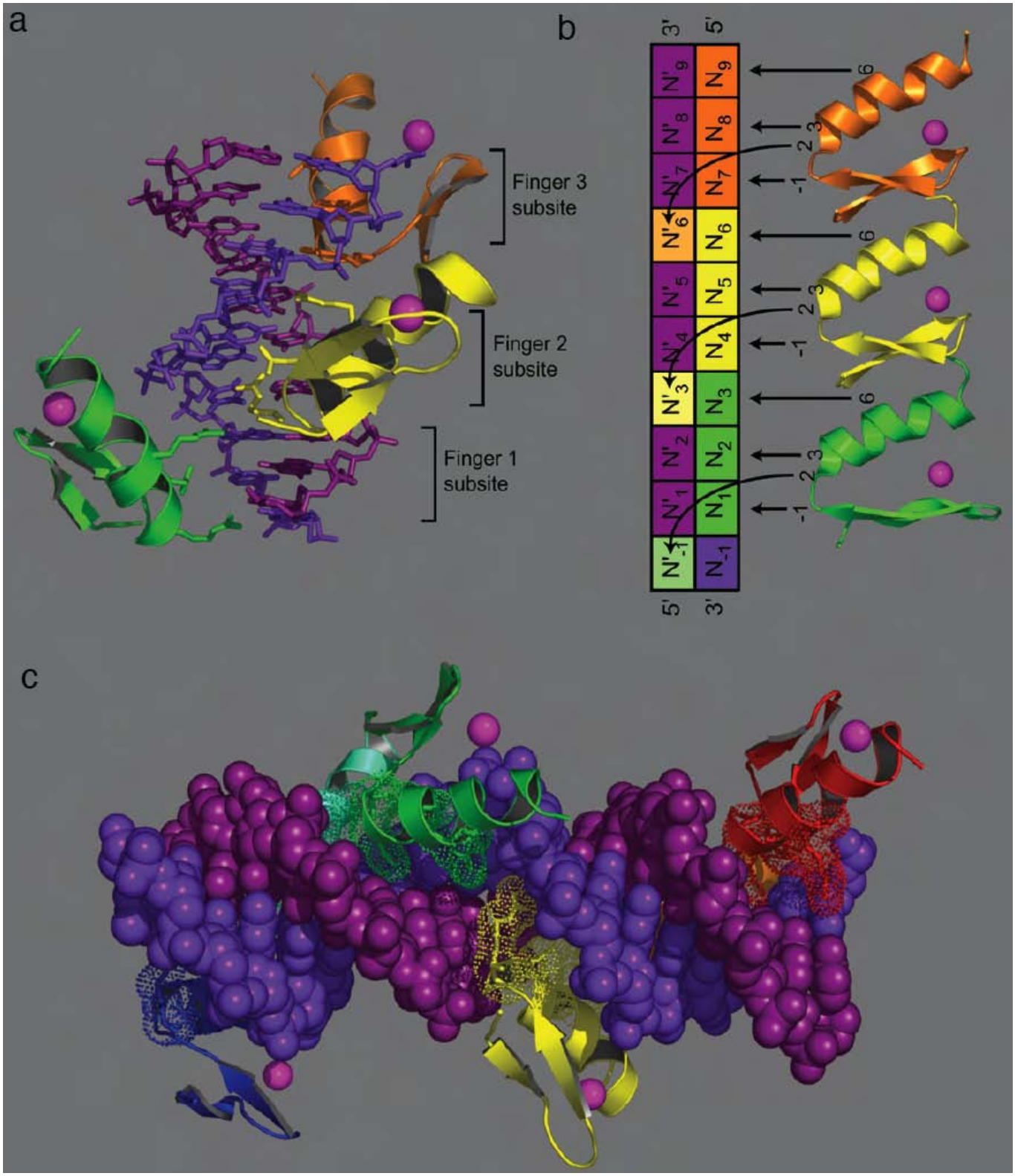

Fig. (2). Modular assembly of ZF proteins. (**a**) Crystal structure of a three-ZF protein Zif268 bound to DNA [1]. (**b**) Model of DNA recognition of a three-ZF protein with a 9-bp DNA substrate. (**c**) Molecular model of a designed 6ZF protein binding the DNA major groove.

domains with distinct DNA-binding capabilities [1-2, 20]. The initial experiments were performed using the phage display technique by multiple laboratories [26-31]. In these experiments, ZF libraries were generated on the surface of the bacteriophage (Fig. **3a**). Typically, the recognition α-helix of one of the ZFs of the 3ZF protein Zif268 was randomized. The ZF library comprised a randomization of the N-terminal residues of the recognition helix (positions –1 to

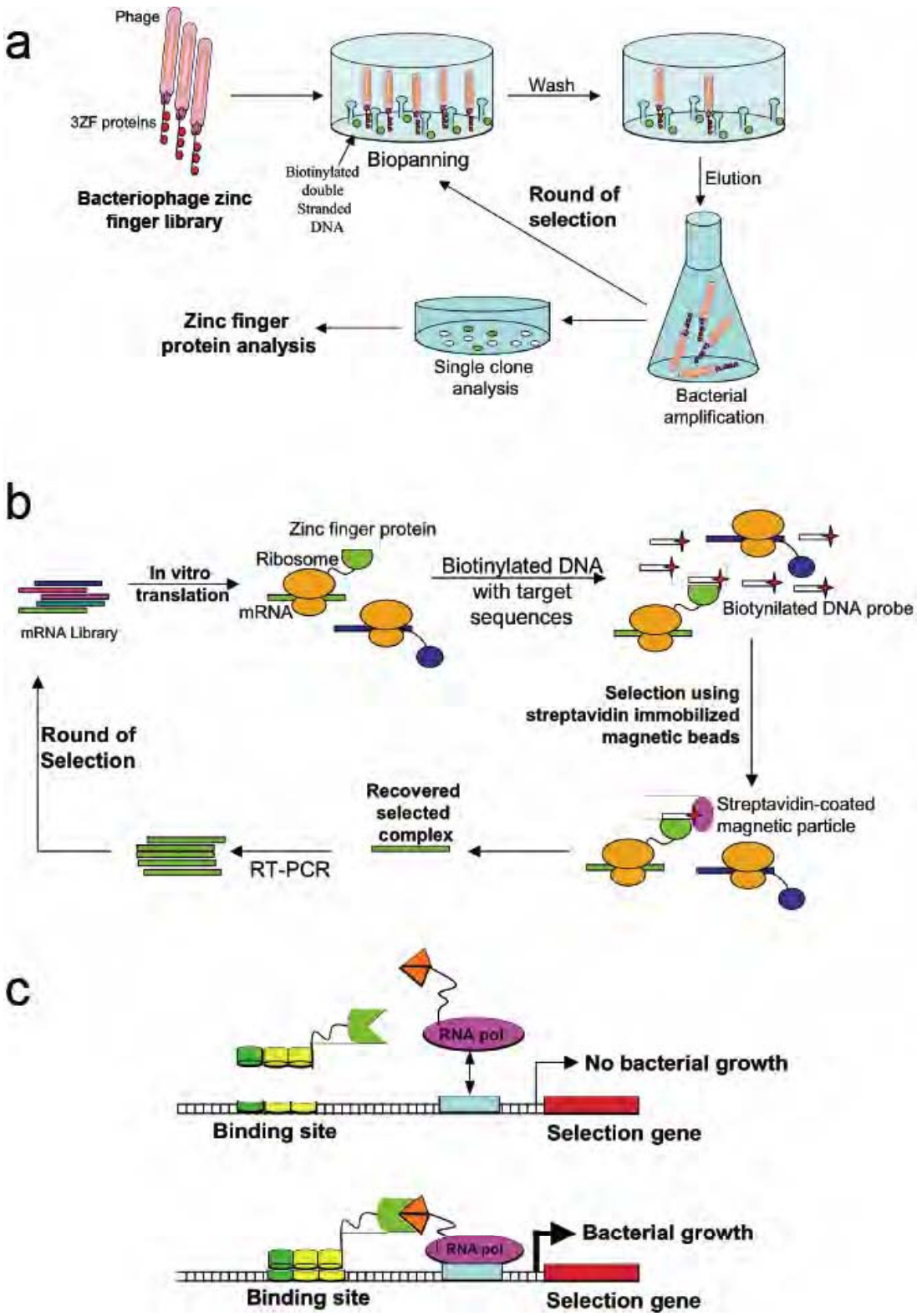

Fig. (3). Strategies for the selection of Zinc Fingers (ZFs) with different DNA-binding specificities. (**a**) *Phage Display*. ZF libraries are generated on the surface of the bacteriophage. The phage display selection is made using biotinylated double stranded DNA comprising particular triplet combinations as targets for the randomized ZF. During the biopanning process, ZF proteins displayed on the phage are allowed to bind to the biotinylated double stranded DNA. Unbound phage is washed out and specific ZF-phages are eluted, amplified in bacteria and used for a second round of selection. Typically several round of biopanning (3-6 rounds) are necessary to amplify specific ZFs. (**b**) *Ribosome display*. A mRNA ZF library is translated *in vitro* to produce mRNA-ribosome-translated proteins. A biotinylated DNA with the target sequence is added to allow the ribosome-translated proteins to bind to the biotinylated probe. The selection is made in streptavidin immobilized magnetic beads, and the complexes are disassembled, amplified by RT-PCR and transcribed *in vitro* for the next round of selection. (**c**) *Bacterial two hybrid systems*. A three-zinc finger protein binds to its cognate DNA binding site, recruiting the RNA polymerase to the promoter and triggering transcriptional activation of the gene. The selection marker is expressed allowing the bacteria to grow in selective media.

+6), which directly contact DNA or are critical for positioning the contact residues with the DNA-bases. The substrates used for the phage selection experiments (biopanning) were biotinylated double stranded DNA oligonucleotides comprising particular DNA triplet sequences as targets for the randomized ZF. The other two ZFs were not randomized and served to "anchor" the protein by binding their corresponding triplets in the target DNA. During the biopanning process, typically 10^{11} phage library members were incubated with beads coated with the target double-stranded oligonucleotides. Unbound phage was washed and specific ZF-phages were eluted, amplified in bacteria and used for a second round of selection. Competitor DNA was used in the binding reaction to ensure that specific ZF phages were selected. Several rounds of biopanning (3-6 rounds) were necessary to amplify specific ZFs. Using this technique several laboratories had identified specific ZF helices interacting with all 5'-GNN-3' [27, 29], most of 5'-ANN-3'[30] and 5'-CNN-3'[31] triplets, were N=A,C,G,T. Due to library size restrictions, most selections performed using this methodology used a randomized ZF1 or ZF2 in the Zif268 backbone [32-34]. These experiments demonstrated that interactions between the contact positions –1, +3, +6 and the corresponding DNA bases of the recognition triplet could be rationalized, as described by molecular modeling and structural studies of families of Zif268 variants selected by phage display [1-2]. Since each ZF behaves as a quasi-autonomous unit, we can extrapolate the information obtained with a "generic ZF" and apply this to any domain of the protein. As described below, in the modular strategy for the construction of ZF proteins, Barbas and collaborators have implemented a "helix grafting" approach to change the specificity of multimodular ZF proteins. In this approach, one can design any ZF by simply exchanging or "grafting" the recognition α-helix of this particular ZF using the generic information obtained by phage display and further refined by mutagenesis experiments [33, 35-37].

As expressed previously, ZFs do not completely function as autonomous domains, hence there are inter-domain contacts between the ZF α-helices and the corresponding DNA-triplets, a phenomenon also described as "target site overlap". For example, position +2 of the α-helical domain of a given ZF can potentially bind the 3' nucleotide of a DNA triplet in the complementary strand (Fig. **2a**), which is the binding site recognizing the previous ZF domain. Thus, the context-dependence of the assembly could potentially modify the predicted specificity of the resulting protein.

Ideally, an optimized multifinger library would involve the simultaneous randomization and subsequent selection of each ZF α-helix of the protein for each DNA-targeted site. However, this would generate very complex libraries with more than 10^{24} clones, which are impossible to screen either by phage display or by cell-based reporter assays. To address this challenge, several strategies have been developed. Greisman and Pabo have developed a phage display strategy based on the sequential randomization and phage selection of one ZF at the time [38]. However, this sequential selection is tedious and difficult to perform by a laboratory today and not suitable for high-throughput screening assays. In order to overcome the target site overlap Choo and collaborators implemented a phage display base bi-partite strategy [39]. This approach involves the construction of two complementary master libraries using the Zif268 framework. The libraries are used in parallel to select for two DNA-binding domains each recognizing 5-bp sequences of a 9-bp target sites. The results of phage display experiments from both libraries are then recombined to generate single clones [39].

Recently, a modification of the ribosome display technique has been described for the isolation of Zif268-variants recognizing the cognate Zif268 DNA sequence, using a cell-free, *in vitro* translation system [40]. Randomized libraries of Zif268 ZFs are first translated *in vitro*. The ribosomes presenting the ZF libraries are bound to biotinylated DNA substrates. After selection with streptavidin-coated magnetic beads, the ZF-coding sequences are amplified by reverse transcription and PCR. Although some Zif268 variants recognizing its cognate DNA site have been selected using this method [40], no published data is yet available regarding the selection of ZFs binding heterologous DNA triplets (Fig. **3b**).

In another report, a direct "read out" of ZF-DNA interactions was performed directly in a bacterial lysate by expression of the ZF libraries fused with GFP with simultaneous biopanning with the DNA-target site [41-42]. Binding was measured in a high-throughput system by measuring GFP in a plate reader. The advantage of this system is that it does not require purification of ZF proteins and allows a direct measurement of ZF-DNA interactions. Using this strategy, several ZF libraries were created by randomizing one amino acid residue at the time, similarly to the Houghten's positional fixing methodology in peptide screenings [43].

Other methods to select for optimized arrays of ZF units binding specific targeted DNA sequences are performed in living cells. Hurt *et al.* [44] have isolated and optimized ZF proteins by a directed domain shuffling and selection in bacterial cells using a bacterial two-hybrid system (Fig. **3c**) [45]. In this system, individual ZF libraries are randomized using a plasmid expressing the 3ZF DBDs of Zif268 in fusion with the Gal11P protein, which recruits the bacterial RNA polymerase. A target plasmid contains the ZF-binding sites driving expression of a reporter gene (typically His3 or LacZ). When the two plasmids are co-transfected in bacterial cells and a functional DBD from the library is produced the transactivation of the reporter gene allows the selection and semi-quantitative quantification of DNA-protein interactions. In the Hurt method individual selections of ZF1, ZF2 and ZF3 are performed in bacteria and the pools of the 3 independent ZF selections are then randomly recombined and re-selected at a higher stringency to generate optimized multimodular ZF DBDs [44]. More recently, a modification of the bacterial one hybrid system has been adapted to study the specificity profiles of multimodular ZF proteins by using an ATF-expressing plasmid carrying the ZF DBDs and target plasmids comprising randomizations of the ZF binding sites [46]. Thus, this novel method allows an *in vivo* high-throughput profiling of multifinger sites at DNA-level.

RATIONAL DESIGN OF ZF PROTEINS BY A MULTIMODULAR APPROACH: CONSTRUCTION OF ZF PROTEINS FOR TARGET GENE REGULATION

Many laboratories, including our own, have successfully applied the "helix grafting" strategy or "modular approach" to generate 3- and 6ZF proteins with many different

specificities [35-36]. The advantage of this approach is that it is quick and easy to apply with conventional molecular biology techniques by any laboratory today (Fig. **4**).

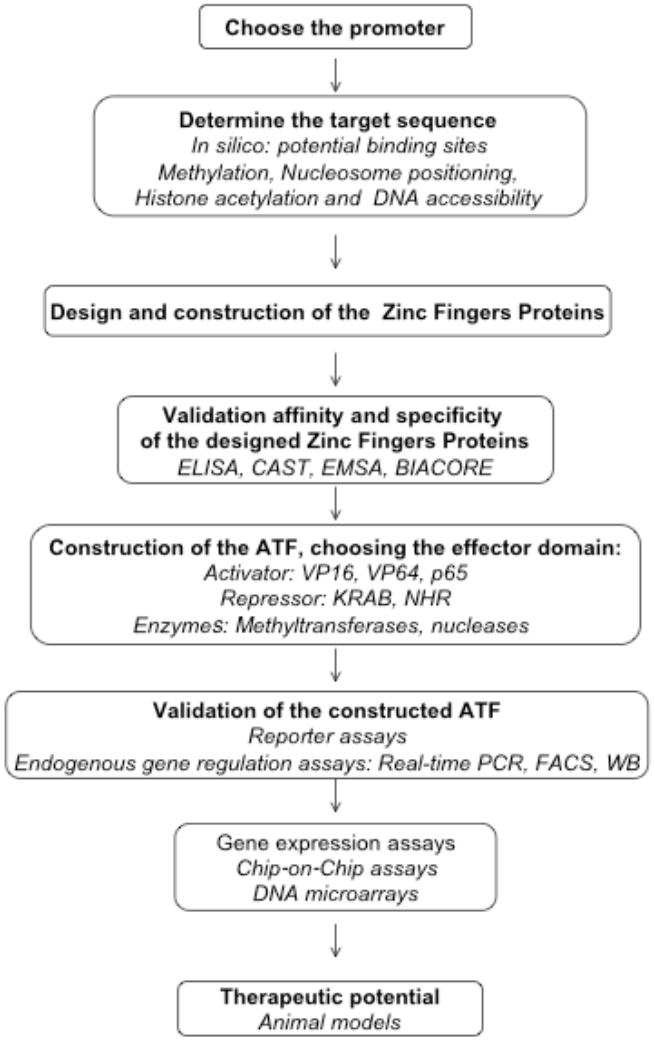

Fig. (4). A design method to generate custom zinc finger proteins. A general protocol can be followed to design, construct and validate successful zinc finger (ZF) proteins against any target gene.

In the *helix grafting approach,* the first step involves the identification of potential binding sites for the multimodular ZF protein in a targeted locus. As shown in Table **1**, designed ATFs binding 18-bp sequences in the gene promoter and also in the 5' UTR have been used to regulate most of the target genes. Given the size of a complex genome, such as human, mouse or Arabidopsis, a 6ZF protein targeting an 18-bp sequence, assuming perfect specificity, can potentially bind single sites [47]. Indeed, recent microarray data has demonstrated that 6ZF proteins are able to alter single genes [48]. In addition, due to their increased number of H-bonding interactions, 6ZF proteins generally have better affinities than 3ZF proteins [36]. For these reasons, the vast majority of multimodular ATFs have been engineered with 6ZF building blocks (Table **1**).

Two web-based tools are now available for designing multimodular ZFs: Zinc Finger Tools, http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php [49] and Finger Targeter, ZiFiT http:/bindr.gdcb.iastate.edu/ZiFiT/ [50]. Given a sequence of DNA in a targeted region of the genome, these web-based tools generate designed ATFs based on the information available for the existing ZF lexicons publicly available by several sources, including published data and the Zinc Finger Consortium. In Zinc Finger Tools, the ZF "solutions" for a given DNA target site promoter are linked with *in vitro* specificity data. It is important to note that for a given triplet many ZF helices might be available (the ZF code is "degenerate") and thus, the *in vitro* information is crucial in order to choose the most "a priori" specific ZFs for the multimodular construction of ATFs [35-36]. Second, several ATFs (two to three are recommended) need to be constructed to target a given promoter region, hence, in the endogenous gene, only a few ATFs could have activity due to chromatin restrictions [51].

The constructed ATFs can be characterized first by *in vitro* affinity and specificity data, such as multitarget ELISA, Electromobility Shift Experiments (EMSA) and Cyclic Amplification of Selected Targets (CAST) assays [36]. The last assay interrogates a random library of dsDNA oligonucleotide substrates for a given multimodular ZF produced *in vitro*. Data derived from CAST assays from over a hundred ZF proteins binding 5'-GNN-3' triplets and constructed using the modular approach have demonstrated that the majority of designed ZF proteins retain their "expected specificity" [36, 52]. However, investigators are encouraged to verify specificity *in vitro* by interrogating their proteins with binding assays such as CAST and ELISA, especially for ATFs comprising non-GNN triplets. A recent crystal structure of a designed 6ZF protein binding 5'-ANN-3' triplets has revealed non-canonical interactions between the ZF α-helices and the DNA [53]. Indeed, particular assemblies of ZFs could yield non-expected interactions due to the context dependence of the ZF assembly.

Multiple genes have been targeted using a multimodular approach, including cellular oncogenes [33, 35, 52], tumor suppressors [51] and viral proteins [54]. These data are summarized in Table **1**. Most targets have been perturbed at the level of transcription by attaching the DBD with a variety of activator or repressor domains. As described below, the DBD has recently been linked to chromatin modifying domains [55-57]. Therefore, compared to RNAi and cDNA delivery, distinctive features of the ATF technology include its versatility of applications, not only to up/down regulate DNA but also to directly modify the chromatin.

The function of the resulting multimodular ZF proteins has been proved *in vitro*, in cellular and animal model systems. Of particular importance are the ZF proteins designed to regulate the *Vascular Epithelial Growth Factor* (*VEGF*) gene [57-58], which are presently in clinical trials [58]. The genomic specificity of the ATFs can be validated by genome-wide gene expression analyses, including DNA-microarray experiments [48, 59] and chromatin immunoprecipitation (ChIP) assays [60]. DNA-microarray experiments will reveal the group of genes regulated by the ATF but these will include both "direct" and also downstream targets of the ATF [59]. ChIP assays will help investigators define the collection of targets directly bound by the ATF. Genome-wide *in vivo* mapping of natural TFs, such as Rap1, has

Table 1. Endogenous genes regulated by Artificial Transcription Factors

Gene	Function	Target Localization	Number of Fingers	Species	Effector Domain		Ref.
Oct-4	Transcription factor	Promoter	6	Mouse	Activator	Repressor	[78]
ErbB-2	Oncogene	5-UTR	6	Human	Activator	Repressor	[33]
PPARg	Cellular differentiation	Promoter	6	Mouse		Repressor	[118]
Epo	Erytropoiesis induction	Upstream	3	Human	Activator		[106]
KRAB-PBS	HIV-primer binding site	5-UTR	6	Human		Repressor	[54]
VEGF-A	Angiogenesis induction	5 and 3 -UTR	3	Human	Activator		[107]
Ap3	Flower development	5-UTR	6	Arabidopsis	Activator	Repressor	[80, 119]
CKH2	Cell cycle	Promoter	6	Human		Repressor	[48]
ADH	Alcohol dehydrogenase	Promoter	3	Arabidopsis	Activator		[120]
Ftz	Transcription factor	Promoter	3	Drosophila	Activator	Repressor	[121]
Maspin	Tumor suppressor	Promoter	6	Human	Activator		[51]
EGP-2	Epithelial glycoprotein 2	Promoter	6	Human		Repressor	[129]
At4CL1	Lignin biosynthesis	Promoter	6	Arabidopsis	Activator	Repressor	[122]
MDR1	Multidrug resistance protein	Promoter	5	Yeast		Repressor	[112]
ErbB-3, ErbB-2	Oncogene	Promoter	6	Human	Activator	Repressor	[35]
CCK2	Gastric acid secretion	Promoter	6	Human	Activator		[123]
ErbB-2	Oncogene	Promoter	3	Human	Activator		[52, 105]
CDH5	Vascular endothelial cadherin	Promoter	3	Human	Activator		[60]
ICAM-1	Intracellular adhesion molecule	Promoter	6	Human	Activator		[52, 60]
	Endonuclease		2 X 3	N/A			[125]
	Recombinase		2 X 3	N/A			[85, 86]
	Methytransferases		3	N/A			[55, 56, 131]

revealed that proteins bind DNA at both upstream coding regions but also in intergenic sequences. The binding of Rap1 involves approximately 5% of yeast genes, targeting 294 loci [61]. Similarly, it is estimated that a 3ZF endogenous TF such as SP1 would bind in the order of 12,000 sites in the human genome [62]. Nevertheless, a binding event might not have influence in transcription as most sequences in the genome don't have regulatory potential. For a highly specific TF, such as a 6ZF ATF, it is expected to have many fewer binding events in the genome. The *integration* of genome-wide ChIP-on-chip experiments [62-65] and bioinformatics approaches (such as ProSITE [66-67] using the predicted ATF-binding site as a probe) is perhaps the most accurate way to evaluate the genomic specificity of a given ATF. However, genome-tiled arrays required for genome-wide ChiP-on-chip assays are fairly expensive and involve specialized technology, perhaps explaining why these experiments have not extensively been used for the study of *in vivo* ATF-DNA interactions. Nevertheless, investigators are strongly encouraged to analyze the genome-specificity of their constructed ATFs as some 6ZF proteins could have "off-target" effects. The recent development of genome-wide chromatin immunoprecipitation assay linked to ultrahigh-throughput DNA sequencing (ChIPSeq [68-70]) could be applied for the future mapping of ATF-DNA interactions. In addition, the integration of novel methods for the generation of genome-wide chromatin structure maps [71-72] with gene expression profiles could help investigators to predict functional ATF-DNA interactions, as it has been described for the TF Myc [73]. As high-throughput mapping of chromatin structure in promoters becomes available for many cells lines, investigators could use this information to target ATF-binding sites.

EFFECTOR FUNCTIONS LINKED TO THE DNA-BINDING-DOMAIN

Many effector domains (EDs) and enzymatic activities can be engineered in conjunction with the designed DBD. Typically, these include specific activation or repression of particular cellular targets, and also, an emerging body of applications takes advantage of modification of the genome or "genome editing".

Some of the most common EDs used for gene activation is the herpes simplex virus VP16 activation domain, its engineered tetramer VP64 [33], and the p65 domain of the transcription factor NFkB [74]. In these designs the activator associates with the mediator protein, which interacts with co-activators and with the polymerase-II transcriptional complex and associated enzymes, facilitating transcription. Some of the enzymatic activities of this complex include Histone Acetyltransferases (HAT) [75-76] and the SWI/SNF [77] chromatin remodeling enzymes. In contrast, repressor EDs like the Kruppel associated box (KRAB) [33, 78], the ligand-

binding domain of thyroid hormone receptor alpha or its viral relative, vErbA [57], the mSin3 interaction domain (SID) and the ERF repressor domain [79-80], interact with transcriptional co-repressors and chromatin condensing enzymes, resulting in decreased accessibility of the promoter for TFs and the polymerase complex [81, 82]. Recently, several groups have achieved transcriptional silencing by attaching the ZF DBDs with a methyltransferase domain (Table **1**). An attractive feature of this approach is that the incorporation of this epigenetic modification confers stably inherited gene silencing [56]. Methyltransferases catalyze the incorporation of methyl groups in position 5 of a cytosine base in CpG islands. The methylated CpG islands are bound *in vivo* by methyl-binding-proteins (MBPs) which are part of a repressive complex containing histone deacetylase (HDCA) and methyltransferase enzymes. As a result, the promoter structure becomes inaccessible to the TFs and DNA-polymerase [83-84]. Other recent applications of engineered ZF DBDs include the ability to specifically recombine and cleave DNA using recombinase [85-86] and Fok-1 nuclease domains [87-88]. The ZF linked to a Fok-1 domain has become a promising tool for homologous recombination. In this design, two chimeras composed by a ZF linked to a Folk-1 domain have to be constructed and must dimerize properly in order to cleave the DNA.

An emerging application of designer DBDs is the *in vivo* imaging of DNA-protein interactions. Two designed 3ZF domains targeting contiguous 9-bp sites are linked to a split-GFP molecule [89] or to a TEM-1 β-lactamase enzymatic protein domain [90]. When the two 3ZF moieties bind their contiguous target sites, a functional GFP or enzymatic function is restored [91]. Even though these experiments have been performed in reporter and in *in vitro* assays, they have the potential to be applied in the context of the genome. Thus, this would provide a unique methodology to assess changes of chromatin structure or accessibility of DNA-binding sites for TFs in real-time on a single cell level.

Our laboratory has investigated the importance of chromatin structure in ATF-mediated regulation of endogenous genes. We have targeted the tumor suppressor gene *mammary serine protease inhibitor* (*maspin*), which acts as tumor and metastasis suppressor for many malignancies, including breast, prostate and lung cancer [92]. The *maspin* gene is found silenced in metastatic tumors but unlike other tumor suppressors, the gene is not found mutated or rearranged in tumor cells [93]. Instead, the *maspin* promoter is found in a dormant state in metastatic cells by means of epigenetic mechanisms, comprising DNA-methylation and transcriptional repression (Fig. **5**). We have constructed 6ZF ATFs binding 18-bp sites along the *maspin* proximal promoter [51]. We found that the efficiency of ATF regulation highly depended on the epigenetic status of the promoter. In cells lines carrying a non-methylated promoter, such as the poorly aggressive MDA-MB-468 breast cancer cell line, two proximal ATFs, ATF-97 and ATF-126, were both able to up-regulate the promoter. However, in the context of a methylated *maspin* promoter, as in the MDA-MB-231 cell line, only one ATF, ATF-126, was able to substantially regulate the promoter. We found that the up-regulation achieved by ATFs was inversely correlated with the degree of DNA-promoter methylation. Methylated DNA in CpG islands act as a substrate for methyl-binding proteins, which are associated with several enzymatic activities, incuding methyltransferase and histone deacetylase. As a result, the promoter becomes inaccessible to TF binding [94-96]. Thus, our results strongly suggest that the structure of the chromatin, which is dynamically controlled by modifications such as DNA and histone methylation and acetylation, could physically restrict the access of ATFs to their target binding sequences.

Recent advances in the field of epigenetics have outlined the importance of the histone modification "code" in gene transcription [97]. Histones are subjected to a vast array of post-transcriptional modifications, including methylation, acetylation, ubiquitination, ADP-ribosylation, sumolation and phosphorylation. Certain modifications, such as acetylation of Histone 3 and Histone 4 (H3 and H4) or di- or trimethylation of H3K4 are associated with active transcription and are referred to as euchromatin modifications. Modifications localized in inactive regions, such as H3K9me and H3K27me are often designated as heterochromatin modifications. Most modifications are distributed in distinct patterns in the upstream region, core promoter, 5' end of an Open Reading Frame (ORF) and at the 3' end of an ORF. Indeed, the exact combination of chemical modifications both at DNA and histone level is highly regulated and could influence the physical access of ATFs and the concomitant recruitment of the DNA-polymerase complex [98]. For example, specific methylation is regulated both by methyltransferases and specific demethylases [99]. This complex "language" of chromatin modifications is rather dynamic, and could be subjected to change in a given cell in response to different stimuli and also could change during disease stages and in between cell types, dictating the transcriptional output. In addition, recent data also demonstrates that nucleosome-positioning in the chromatin is also a dynamic process [98, 100-101]. The major structural component of chromatin, the linker histone H1 and its variants, which are associated with more than 80% of the nucleosomes in a mammalian nucleus, have variable residence time, ranging between 1-3 min in interphase. Residence times are highly variable and subjected to changes depending on the cell cycle and several competing factors [98]. These include TFs and remodeling enzymes [102], which modify or mobilize the nucleosomes, a process referred as nucleosome eviction [98, 103]. Hence, nucleosomes could interfere with ATF-binding under the proper physiological conditions and in particular cell types. This could also be subject to change under the proper regulatory cues. On the other hand, ATFs could *per se* act as destabilizing factors for nucleosomes, promoting nucleosome eviction. Importantly, very little information is available as to how transcriptional gene complexes and chromatin are associated in the 3D space of the nucleus. Recent advances have shown that chromosomes undergo an extensive network of communication, and genes could be regulated by "TF factories" both *in cis* (within the same regulatory region) and *in trans* (interaction with a closely localized region of another chromosome) [104]. In conclusion, a main determinant of ATF-specificity *in vivo* is the structure of the chromatin, and now we understand ATF-DNA interactions as rather plastic events, changing depending on the physiologic state of cells and tissues, and subjected to chromatin "rules". Thus, a more complex and futuristic ATF design will involve the targeting or the modification of the chromatin, both at histone and DNA level, to fa-

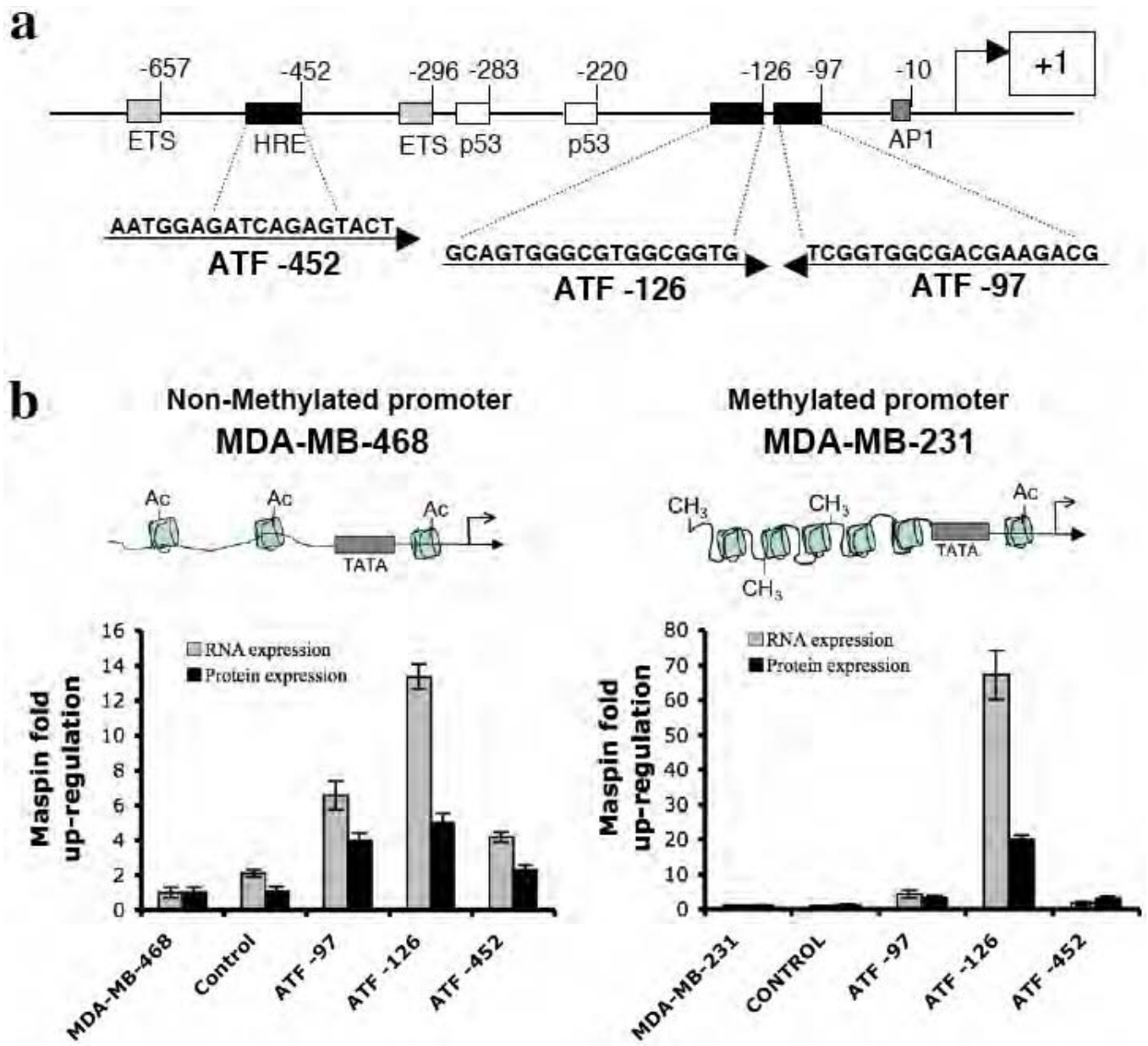

Fig. (5). Influence of chromatin in ATF-regulation. (**a**) *Maspin* promoter showing the sequence and binding sites of ATF-97, ATF-126 and ATF-452 Artificial Transcription Factors. Some of the response elements for known transcription factors are indicated [51]. (**b**) ATFs regulate *maspin* expression in MDA-MB-468 and MDA-MB-231 breast cancer cell lines. Both, ATF-97 and ATF-126 are able to up-regulate *maspin* in MDA-MB-468. However, only ATF-126 restores *maspin* expression in MDA-MB-231, a cell line carrying a methylated *maspin* promoter. Breast cancer cell lines were transduced with a retroviral vector expressing ATF-97, ATF-126, ATF-452 and a control (empty retroviral vector).

cilitate the proper ATF-access on specific targeted sequences on the DNA. This will be possible as more information becomes available to understand the intrinsic epigenetic grammar at given promoter sites.

SCREENING AND PHENOTYPIC SELECTION OF ATF-LIBRARIES

As expressed above, one major factor that restricts the *in vivo* activity of ATFs is the chromatin structure. This has inspired the development of ATF-libraries, or combinatorial repertoires of ZFs, which are shuffled to generate many DNA-binding specificities, in the order of 10^4-10^8 different DNA-binding specificities (Fig. **6**). ATF libraries have been generated with both phage display-selected [33, 36, 52, 105] and endogenous ZF domains [106-108]. The size of the library depends on the number of ZF blocks that are recombined. For example, our 3ZF ATF libraries comprise approximately 10^4 members, whereas than 6ZF libraries comprise 10^8 members. The resulting recombined ZF domains are linked to different effector domains, such as activators or transcriptional repressors. When delivered in complex genomes, the ATF-libraries induce genome-wide experimental perturbations of gene expression. Given the complex size of the libraries, particularly 6ZF libraries comprising approximately 10^8 members, any gene in a given genome has the potential to be up- or down-regulated. ATF pools have been

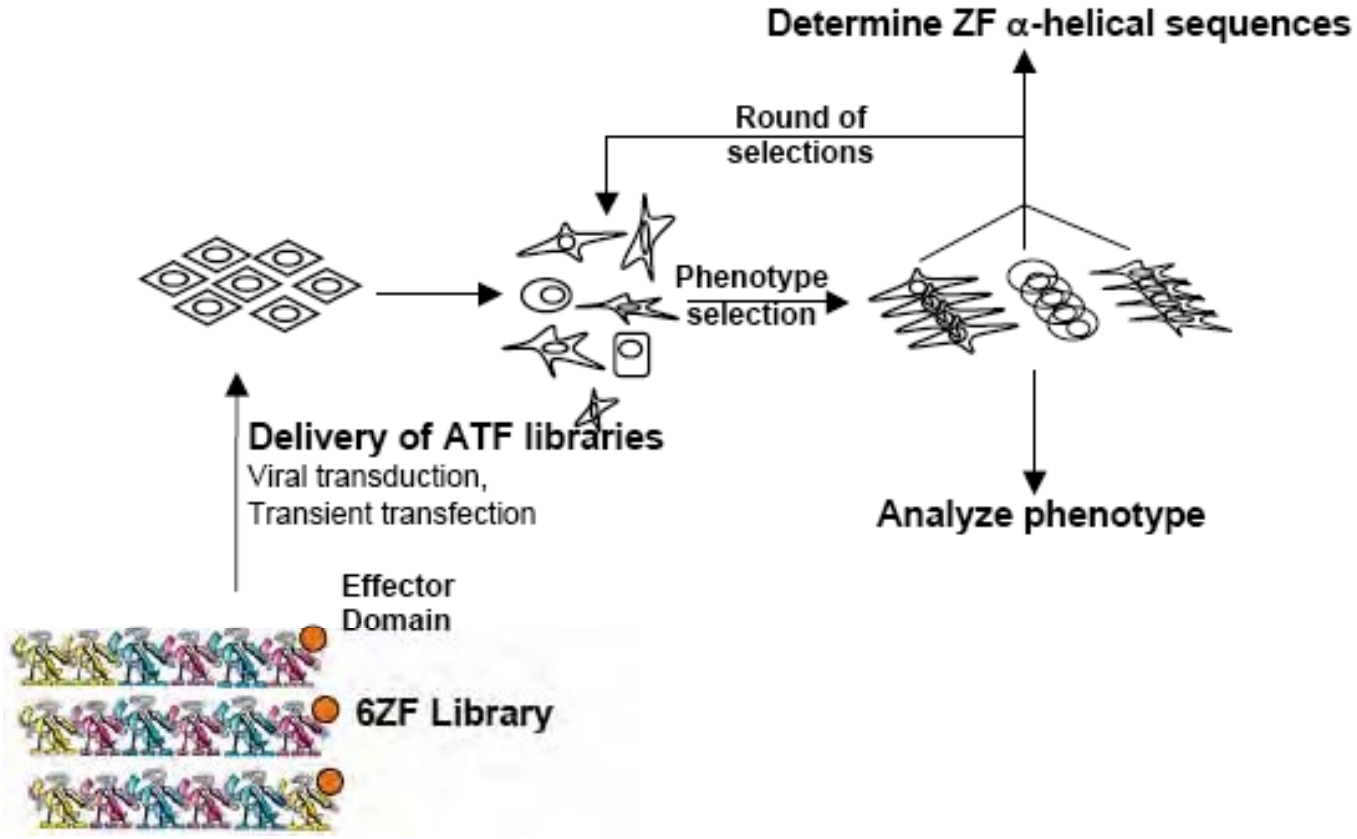

Fig. (6). Endogenous zinc finger (ZF) library selections in mammary cells. The zinc finger libraries are linked to an effector domain and delivered into cells using viral transduction or transient tranfection. The resulting ATF-expressing cell population displays phenotypic plasticity. Next, a phenotypic screen is performed. Specific phenotypes are expanded in subsequent rounds of selection. Genome-wide expression analyses are performed next to evaluate the genes contributing to phenotype specification.

delivered in complex systems, such as bacteria [109-110], yeast [111-113], mammalian cells [37, 52, 59, 60], or even in plant cells [114-115]. ATFs can be delivered using either transient or integrative expression vectors. Retroviral libraries are particularly interesting since they can transduce a variety of mammalian cell types, specially transformed cell types, and since they integrate in the genome they induce stable phenotypes. Upon delivery, the ATF libraries can induce a myriad of phenotypes. We have recently reviewed several phenotypic screens used to identify ATFs from combinatorial libraries [12, 126]. For example, ATF-libraries have been used to induce neoplastic disease progression phenotypes [59] and neural differentiation [127]. In yeast, ATF-libraries induced phenotypes involved in drug resistance [124, 126, 127, 130]. In addition, ATF screens are of interest in biotechnology since allow the isolation of yeast and mammalian cells, which increase the production of recombinant proteins [128].

As shown in Fig. **6**, the cells carrying the phenotype of interest can be propagated. The ATF is then isolated and used as a molecular probe to dissect genes involved in complex phenotype specifications. Complex ZF libraries such as 6ZF libraries have the advantage to be potentially more specific in the genome than smaller ATF libraries, such as 3ZF libraries. However, complex ATF libraries require high-throughput screening assays to identify phenotypes, and that can be done for example in 96 screening wells. With the development of robotic high-throughput screening microscopes [116-117] cells displaying a particular phenotype can be isolated. The ATF-encoded DNA is recovered from the cells and additional screenings are performed to isolate ATFs having the desired activity. Single clones of ATFs are next tested for biological activity. Several assays such as differential DNA microarray, bioinformatics searches, and ChIP assays can be performed to evaluate the direct genes that are targets of the ATFs. The sequencing of the ATF provides information of the potential targets of the ATF on the genome and allows the mapping of the ATFs in given promoters.

We have recently performed genomic screenings of ATFs in tumor cells and selected ATFs able to induce complex phenotypes, such as multidrug resistance [59, 126] and induction of metastatic behavior [59]. The selected ATFs can be used to identify novel biomarkers of disease progression and to identify genetic networks, which are involved in complex phenotypes.

In conclusion, ATFs represent a valuable technology to re-program complex phenotypes in cells and tissues by modification of gene transcription in one particular locus or by altering the entire genetic transcriptional network of cells. Other innovative applications of this new technology involve the modification or editing of the genome and the imaging of protein-DNA interactions in living cells. Further development of this technology will involve the generation of viral and non-viral delivery systems for the expression of the

ATF-encoded DNA or the ATF protein in the proper cell type, tissue or organ. Additionally, more investigations need to be performed to study the genome-wide specificity of the ATFs in a given cell type. In the immediate future, ATFs could be applied in molecular therapeutics for the treatment of a variety of complex diseases and phenotypes, including angiogenesis, tumor cell growth and metastasis.

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ABBREVIATIONS

ZF = Zinc Finger

CAST = Cyclic Amplification of Selected Targets

EMSA = Electromobility Shift Experiments

WB = Western Blot

bp = Base Pair

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SHORT COMMUNICATION

Re-activation of a dormant tumor suppressor gene *maspin* by designed transcription factors

A Beltran[1], S Parikh[1], Y Liu[1], BD Cuevas[1], GL Johnson[1], BW Futscher[2] and P Blancafort[1]

[1]*Department of Pharmacology and the Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA and* [2]*Department of Pharmacology and Toxicology, Arizona Cancer Center and College of Pharmacy, University of Arizona, Tucson, AZ, USA*

The controlled and specific re-activation of endogenous tumor suppressors in cancer cells represents an important therapeutic strategy to block tumor growth and subsequent progression. Other than ectopic delivery of tumor suppressor-encoded cDNA, there are no therapeutic tools able to specifically re-activate tumor suppressor genes that are silenced in tumor cells. Herein, we describe a novel approach to specifically regulate dormant tumor suppressors in aggressive cancer cells. We have targeted the *Mammary Serine Protease Inhibitor* (*maspin*) (*SERPINB5*) tumor suppressor, which is silenced by transcriptional and aberrant promoter methylation in aggressive epithelial tumors. Maspin is a multifaceted protein, regulating tumor cell homeostasis through inhibition of cell growth, motility and invasion. We have constructed artificial transcription factors (ATFs) made of six zinc-finger (ZF) domains targeted against 18-base pair (bp) unique sequences in the *maspin* promoter. The ZFs were linked to the activator domain VP64 and delivered in breast tumor cells. We found that the designed ATFs specifically interact with their cognate targets *in vitro* with high affinity and selectivity. One ATF was able to re-activate *maspin* in cell lines that comprise a *maspin* promoter silenced by epigenetic mechanisms. Consistently, we found that this ATF was a powerful inducer of apoptosis and was able to knock down tumor cell invasion *in vitro*. Moreover, this ATF was able to suppress MDA-MB-231 growth in a xenograft breast cancer model in nude mice. Our work suggests that ATFs could be used in cancer therapeutics as novel molecular switches to re-activate dormant tumor suppressors.

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Correspondence: Dr P Blancafort, Department of Pharmacology and the Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365, USA.
E-mail: pilar_blancafort@med.unc.edu
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Tumor progression is a dynamic process controlled by multiple genetic factors, including oncogenes, which facilitate tumor growth, and tumor suppressors, which negatively regulate tumor growth and progression. Since the discovery of the tumor suppressor p53, more than 15 different tumor suppressor genes have been identified (Sherr, 2003; McGarvey *et al*., 2006). The expression of tumor suppressors is downregulated in tumor cells by means of genetic and epigenetic mechanisms (Baylin, 2005; Zardo *et al*., 2005). Given the importance of tumor suppressors in controlling primary tumor growth, many therapeutic strategies aim to restore their expression in tumor cells. Tumor suppressors silenced by methylation and transcriptional repression can be re-activated by a variety of chromatin remodeling drugs, such as methyltransferase inhibitors (including 5-aza-2′-deoxycytidine, recently approved for therapeutic treatment (Samlowski *et al*., 2005) and histone deacetylase inhibitors (such as suberoylanide hydroxamic acid (SAHA)). These drugs are able to relax the chromatin enhancing the accessibility of the transcription machinery (Garber, 2004). However, potential limitations for the use of these drugs in cancer patients include their toxicity, lack of target specificity and development of acquired drug resistance (Juttermann *et al*., 1994). In this paper, we describe a novel approach to specifically activate tumor suppressors that are epigenetically silenced in tumor cells. We hypothesized that artificial transcription factors (ATFs) designed to recognize specific sequences in the promoter of a tumor suppressor would result in a re-activation of the endogenous gene in tumor cells. In order to test this, we have chosen the tumor suppressor *SERPINB5*, or *Mammary Serine Protease Inhibitor* (*maspin*), as a prototype of a tumor suppressor gene (Zhou *et al*., 1994) silenced by epigenetic mechanisms in aggressive tumor cells. The choice of this target was based on the following characteristics: (1) *maspin* is not mutated or rearranged in tumor cells, but the gene is silenced during metastatic progression. This offers a unique opportunity for therapeutic intervention through specific re-activation of the endogenous gene. *Maspin* silencing involves: (1) transcriptional regulation (by means of TFs such as p53 (Zhou *et al*., 2000), ETS, AP-1 (Zhang *et al*., 1997), hormone receptor (Zhang *et al*., 1997; Khalkhali-Ellis *et al*., 2004) and aberrant promoter methylation

(Domann *et al*., 2000; Futscher *et al*., 2002). (2) High levels of *maspin* are associated not only with reduction of tumor growth (Sager *et al*., 1997), but also decreased angiogenesis (Zhang *et al*., 2000a), cell motility and invasion (Sheng *et al*., 1996; Seftor *et al*., 1998), and metastatic dissemination (Zhang *et al*., 2000b; Shi *et al*., 2002; Cher *et al*., 2003; Watanabe *et al*., 2005). Maspin-mediated reduction of tumor cell growth is at least partially attributed to enhancement of apoptosis (Liu *et al*., 2004; Zhang *et al*., 2005), whereas downregulation of cell invasion is associated with the inhibition of the activity of cell-surface-associated urokinase-type plasminogen activator (Lockett *et al*., 2006; Yin *et al*., 2006). (3) *Maspin* is clinically relevant for several types of human cancers: breast, prostate, colon and squamous carcinomas. For these tumors, *maspin* expression is a predictor of better prognosis (Lockett *et al*., 2006).

Results and discussion

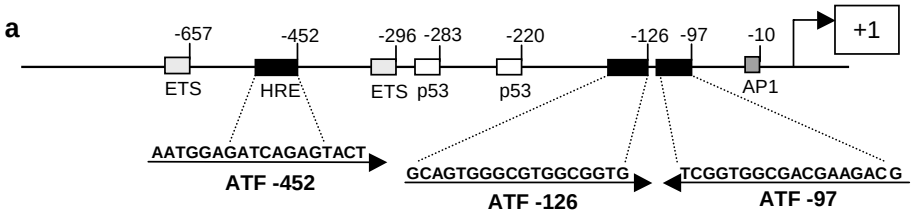
In order to upregulate specifically the *maspin* promoter in breast cancer cell lines, we have designed ATFs made of six zinc-finger (6ZF) domains linked to the VP64 activator domain. Each ZF domain specifically recognizes 3 base pairs (bps) of DNA (Pavletich and Pabo, 1991). Thus, a designed 6ZF ATF will recognize 18-bp, providing high degree of specificity (Blancafort *et al*., 2004; Beltran *et al*., 2006). Indeed, previous reports have described that highly specific 6ZF ATFs are able to target unique sites in the human genome (Tan *et al*., 2003). We chose three 18-bp sites in the *maspin* proximal promoter as targets for our ATFs (Figure 1a). A BLAST-Search on the human genome has revealed that the three 18-bp targeted sites in the *maspin* promoter are unique (data not shown). Targets −126 and −97 were selected based on their close proximity to the transcription start site and because of the highly specific interactions predicted for the DNA triplets and the available ZF lexicons (Segal *et al*., 1999; Dreier *et al*., 2000, 2002, 2005). In addition to the proximal ATF sites, we have designed an ATF recognizing an 18-bp site comprising the hormone response element (HRE) sequence, which interacts with the hormone receptor both in breast (Khalkhali-Ellis *et al*., 2004) and prostate (Zhang *et al*., 1997) cells. It has been shown that treatment of estrogen receptor (ER)+ breast cancer cells with the ER modulator Tamoxifen (TAM) results in the re-activation of *maspin*, and that this effect requires an intact HRE sequence (Khalkhali-Ellis *et al*., 2004). Given the importance of HRE in regulating *maspin*, we reasoned that an ATF activator binding to a sequence comprising the HRE site could also lead to effective *maspin* transactivation.

The 6ZF DNA-binding domains (DBDs) designed to bind the 18-bp targets in the *maspin* promoter were built by overlapping PCR, by grafting the α-helical-coding sequences known to interact specifically with the targeted triplets (Figure 1b; Beerli *et al*., 1998). To verify that the designed 6ZF DBDs were able to interact specifically with their cognate substrates, we first purified the ZFs as C-terminal fusion with the carrier maltose-binding protein (MBP) (Figure 1c). Purified 6ZF-MBP fusions were next tested by a DNA-binding enzyme-linked immunosorbent (ELISA) assay to determine specificity and selectivity (Blancafort *et al*., 2003). We used several double-stranded oligonucleotide variants incorporating single and multiple nucleotide substitutions in the ZF binding sites to assess DNA-binding selectivity. As shown in Figure 1c, the 6ZF-DBDs bound their cognate substrates with high affinity, in the nano- and subnano-molar range. In addition, the designed DBDs appeared to be highly selective *in vitro* since nucleotide substitutions in the DNA-duplex substrate increased the relative dissociation constant (K_d). Single-nucleotide substitutions in all the 6ZF triplets increased the K_d more than 100-fold (Figure 1d). Our results suggested that our designed DBDs bound their predicted substrates *in vitro* with sequence selectivity and in agreement with previous reports (Dreier *et al*., 2000). However, more studies need to be performed to evaluate which degenerated DNA sequences could be bound by the ATFs *in vivo*.

To study if the designed 6ZF DBDs were able to transactivate the *maspin* promoter in breast cancer cells, we first performed reporter assays. The 6ZF DBDs were expressed as fusions with the powerful transcriptional regulator VP64, using a transient expression vector (pcDNA3.1; Blancafort *et al*., 2003). Breast monocyte chemoattractant factor (MCF)-7 cells were co-transfected with the ATF expression vectors and with a reporter vector comprising the luciferase gene driven by the proximal 527-bp *maspin* promoter comprising all the 6ZF binding sites and two p53 binding sites (Zhou *et al*., 2000). Consistent with previous reports, we found that

Figure 1 The designed ATFs are able to bind specifically to their cognate 18-bp sequence and to activate the *maspin* promoter. (**a**) Binding sites for the three designed ATFs in the proximal *maspin* promoter. Numbers designate the distance in bps relative to the start of translation (first ATG codon, +1). Some of the response elements for known transcription factors are indicated. (**b**) α-Helical ZF sequences engineered to bind their corresponding target DNA triplets. These ZF sequences were chosen based on the available specific ZF lexicons (Segal *et al*., 1999; Dreier *et al*., 2000, 2001, 2005). The 6ZF ATFs were constructed by overlapping PCR; Supplementary methods). (**c**) The designed ATFs are able to bind specifically to their cognate 18-bp substrate, as assessed by DNA-binding ELISA (Segal *et al*., 2003; Supplementary methods). The ZF DBDs were expressed as C-terminal fusions with MBP (NEB, Ipswich, MA, USA). These fusions were more than 90% pure as assessed by SDS-PAGE (right panel). (**d**) Relative dissociation constant (K_d) of each protein for their cognate substrate and for different site-directed mutant substrates. An ELISA assay was used to calculate the relative K_d using as standard control ATF 30 (a 6ZF protein of a known K_d as described (Blancafort *et al*., 2003)). (**e**) The designed ATFs transactivate the *maspin* promoter in a luciferase assay in MCF-7 cells. Schematic view of the *maspin* promoter-luciferase construct indicating the ATF- binding sites (Zhang *et al*., 1997) Control represents a non-specific ATF. A p53 expressing plasmid was used as positive control (Supplementary methods). All experiments were carried out in triplicate ± s.d.

expression of p53 in MCF-7 cells was able to upregulate 2.9-fold *maspin* reporter activity (Figure 1e). However, the designed ATFs were able to transactivate 31.2- (ATF-97), 4.1- (ATF-126) and 4.56- (ATF-452) fold the reporter in the same assay. The interaction was specific since the expression of a nonspecific (control) ATF did



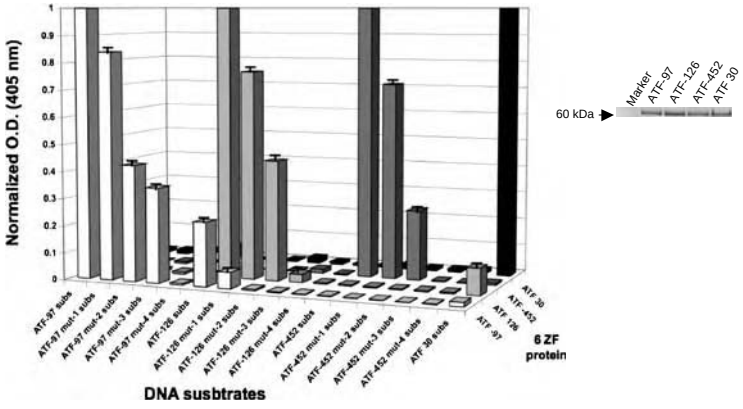
b

ATFs	a) ZF α-HELICES						b) 18-bp TARGET SITES		c) RelativeK_d
	ZF6	ZF5	ZF4	ZF3	ZF2	ZF1	Half-site1	Half-site2	(nM)
ATF -97	QSGDLRR	QSSNLVR	QSGDLRR	RSDDLVR	RSDVLVR	TSGELVR	5′-GCA GAA GCA -	GCG GTG GCT-3′	3.1 ± 0.04
ATF -126	QSGDLRR	RSDVLVR	DPGHLVR	RSDVLVR	RSDDLVR	RSDVLVR	5′-GCA GTG GGC -	GTG GCG GTG-3′	0.9 ± 0.06
ATF -452	TTGNLTV	QSSHLVR	TSGNLVR	RADNLTE	HRTTLTN	THLDLIR	5′-AAT GGA GAT -	CAG AGT ACT-3′	1.74± 0.2

a) ZF (ZF1 to ZF6) helices are positioned in the anti-parallel orientation (COOH-ZF6 to ZF1-NH_2) relatively to the DNA target sequence. For example, for ATF-452, ZF6 recognizes the AAT triplet, ZF5 recognizes GGA; ZF4, GAT; ZF3, CAG; ZF2, AGT; ZF1ACT. The amino acid positions (-1 to +6) in the ZF recognition helix are shown.

b) Target DNA sequences in the *maspin* promoter are presented in the 5′ to 3′ orientation.

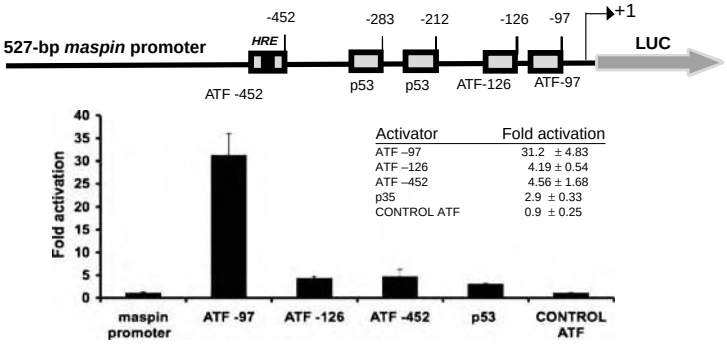
c



d

Protein	Substrate	18-bp TARGET SITES	Relative Kd (nM)
ATF –97	ATF-97 substrate	GCA GAA GCA GCG GTG GCT	3.1 ± 0.04
	ATF-97 mutation 1 subs	GCA **T**AA GCA GCG GTG GCT	6.5 ± 0.22
	ATF-97 mutation 2 subs	GCA GAA GCA **T**CG GTG GCT	7.2 ± 2.3
	ATF-97 mutation 3 subs	GCA GAA **T**CA **T**CG **T**TG **T**CT	7.25 ± 0.68
	ATF-97 mutation 4 subs	**T**CA **T**AA **T**CA **T**CG **T**TG **T**CT	482.6 ± 128.77
ATF –126	ATF-126 substrate	GCA GTG GGC GTG GCG GTG	0.9 ± 0.06
	ATF-126 mutation 1 subs	GCA GTG GGC GTG **T**CG GTG	1.17 ± 0.03
	ATF-126 mutation 2 subs	GCA GTG GGC **T**TG **T**CG **T**TG	2.04 ± 0.32
	ATF-126 mutation 3 subs	GCA GTG **T**GC **T**TG **T**CG **T**TG	28.7 ± 17.55
	ATF-126 mutation 4 subs	**T**CA **T**TG **T**GC **T**TG **T**CG **T**TG	168.3 ± 49.29
ATF -452	ATF-452 substrate	AAT GGA GAT CAG AGT ACT	1.74 ± 0.2
	ATF-452 mutation 1 subs	AAT **T**GA GAT CAG AGT ACT	2.41 ± 0.47
	ATF-452 mutation 2 subs	AAT GGA **T**AT CAG AGT ACT	6.7 ± 3.95
	ATF-452 mutation 3 subs	AAT GGA **T**AT CA**T** AGT ACT	630.8 ± 137.02
	ATF-452 mutation 4 subs	AAT **T**GA **T**AT CA**T** AGT ACT	709.7 ± 111.28

e



not result in significant reporter upregulation. The higher transactivation observed for ATF-97 could be explained by its close proximity to the transcription start site, facilitating the recruitment of the polymerase II complex (Stege *et al*., 2002).

To study if the designed ATFs were able to upregulate endogenous *maspin* in breast cancer cell lines, we expressed the ATFs using the retroviral vector pMX-IRES-GFP (Royer *et al*., 2004), which allows the tracking of transduced cells by flow cytometry. All cell lines were efficiently transduced by the three ATFs, as assessed by flow cytometry (data not shown). Effective ATF expression was also verified by semiquantitative reverse transcription–polymerase chain reaction (RT–PCR), using ZF-specific primers for the detection (Figure 2). To evaluate Maspin expression, transduced cells were processed by Western blotting, using a well-characterized anti-Maspin monoclonal antibody for the detection (Khalkhali-Ellis *et al*., 2004). To quantitatively detect *maspin* mRNA expression in transduced cells, we

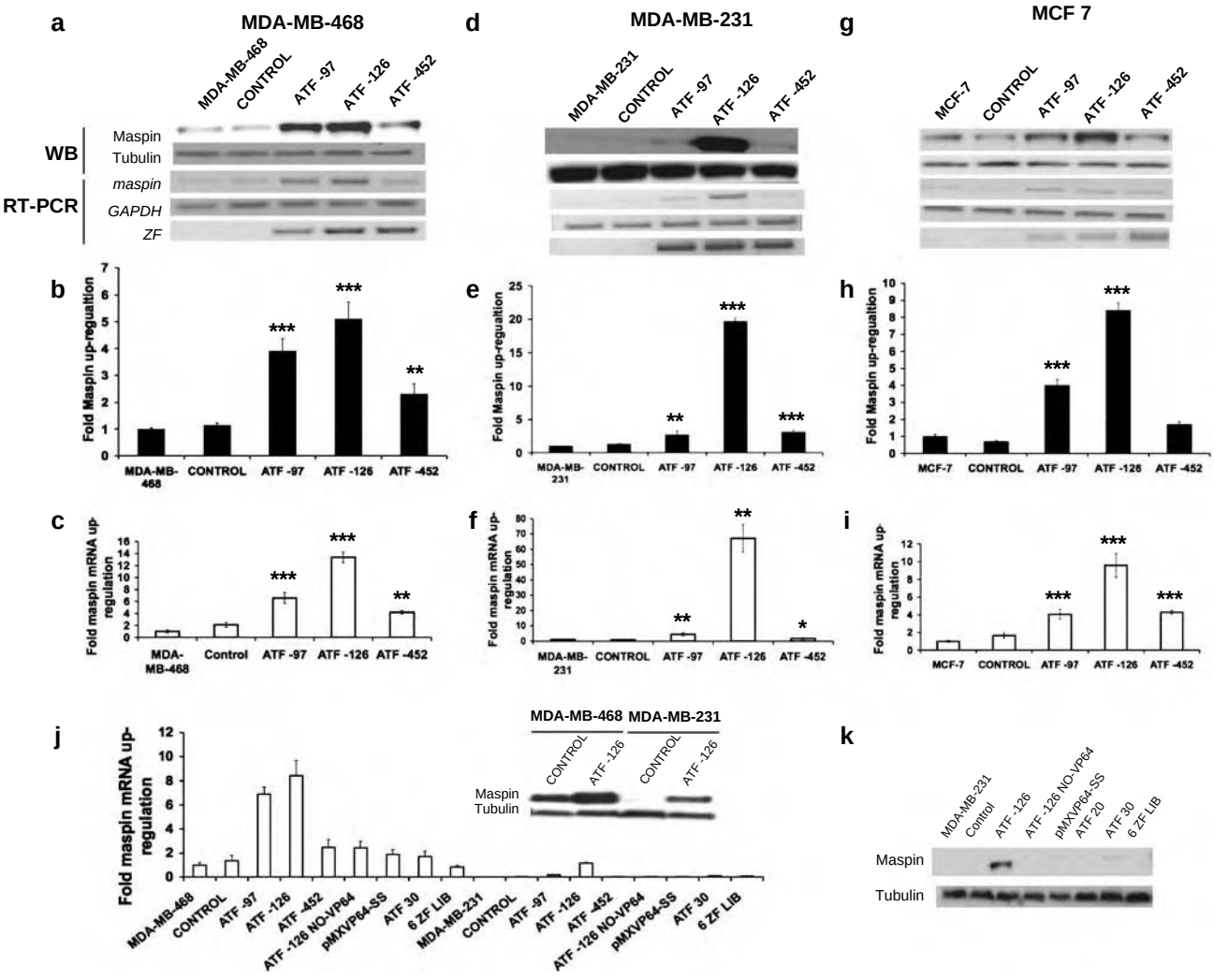


Figure 2 The designed ATFs regulate *maspin* in MDA-MB-468 (**a–c**), MDA-MB-231 (**d–f**) and MCF-7 (**g–i**) breast cancer cell lines. Data were normalized to controls (cells transduced with an empty retroviral vector). Quantification of Maspin protein levels was performed by Western blot (WB) and induction of *maspin* mRNA levels were assessed by real-time expression analysis. ATF's expression in transduced cells was verified by semiquantitative RT–PCR, using specific ZF primers (*ZF* in panels a, d and g). (**j**) ATF-126 restores *maspin* expression in MDA-MB-231 at a level comparable to the endogenous expression of *maspin* in un-transduced MDA-MB-468 cells. MDA-MB-231 and MDA-MB-468 cells were transduced with retroviral vectors expressing ATF-126, a mutant ATF-126 with no VP64 activator domain (ATF-126 NO VP64), ATF-126 without the ZF DNA-binding-domains (pMXVP64-SS), two unrelated 6ZF ATFs (ATF-20 and ATF-30) and a scramble of ATFs (10^8 6ZF library members linked to the VP64 activator domain (6ZFLIB-VP64)). (**k**) ATF-126 specifically regulates *maspin* in MDA-MB-231 cells. The ATFs were cloned in the retroviral vector pMX-SS-VP64-IRES-GFP, which enables the expression of the ZF domains as a fusion with the VP64 activator domain (Blancafort *et al*., 2003; Supplementary methods). After 72 h post-transduction, cells were collected, lysed and processed by Western blotting using an anti-Maspin antibody (1:2000; BD PharMingen, San Diego, CA, USA) and anti-tubulin antibody (Sigma, St Louis, MO, USA). Western blot and real-time quantification is an average of three independent experiments and s.e's are indicated (*$P<0.05$, **$P=0.01$, ***$P=0.001$, as determined by the Student's *t*-test) (Supplementary methods for PCR conditions).

performed real-time expression analysis. We first delivered the ATFs in the MDA-MB-468 cell line, an ER−, poorly invasive, breast cancer cell line, which express *maspin* and comprises a non-methylated *maspin* promoter (Oshiro *et al*., 2003). In this cell line, ATF-97, ATF-126 and ATF-452 were able to upregulate endogenous Maspin (3.3-, 5.4- and 2.3-fold, respectively) relative to controls (cells transduced with empty retroviral vectors, which express Green Flourescent Protein (GFP); Figure 2a–c). This upregulation was specific as the deletion of the VP64 activator domain, the delivery of ATFs with different DNA-binding specificity (ATF-20 and ATF-30) or the delivery of a scramble of 6ZF ATF activator library (10^8 members; Blancafort *et al*., 2003) did not result in any detectable *maspin* regulation (Figure 2j–k). Interestingly, the ATF with stronger activity *in vivo* (ATF-97) was not the stronger activator in the reporter assay. This result suggests that the structure of the chromatin could play a role in ATF-mediated regulation of the endogenous gene. Consistent to this, we found that ATF-mediated regulation depended on the cell line analysed. In the MDA-MB-231 cell line, an aggressive cell ER− breast cancer cell line, which comprises a methylated and silenced *maspin* promoter, ATF-126 was able to strongly re-activate the promoter (70-fold relative to controls), whereas that ATF-97 and ATF-452 had a weak but significant activity (2.95 and 1.93, respectively). Control MDA-MB-231 cells did not express any detectable endogenous Maspin, which was consistent with the reported epigenetic silencing of the promoter in these cells (Figure 2d–f). Importantly, ATF-126 was able to re-activate *maspin* at levels comparable to the MDA-MB-468 cell line, which comprises a non-methylated promoter (Figure 2j). Finally, the ATFs were delivered in the ER+, non-invasive MCF-7 cell line, which comprises a *maspin* promoter silenced by methylation and transcriptional regulation. In this cell line, *maspin* was upregulated by ATF-97 (3.1-fold) and ATF-126 (6.9-fold), compared to controls, whereas ATF-452 had a weak but significant effect (Figure 2g–i). The weak but significant endogenous activity of ATF-452 in both ER− and ER+ cell lines could be explained by the fact that this ATF targets an upstream site, which is either unaccessible or too far from the transcription start site to elicit efficient recruitment of the transcription machinery. These experiments demonstrated that ATFs could be used to re-activate endogenous tumor suppressors silenced by epigenetic mechanisms. Consistent to our results, it has been shown that ectopic expression of p53 in the p53-deficient MDA-MB-231 also results in a partial re-activation of the endogenous gene (Oshiro *et al*., 2003). p53 expression did not alter the methylation status of the *maspin* promoter, but rather increased acetylation levels and *maspin* promoter accessibility, thereby facilitating transcription. Similarly, we could speculate that ATFs could also enhance histone acetylation levels through the VP64 activator domain, resulting in increased chromatin accessibility and enhanced transcription. This is presently being investigated in our laboratory.

We subsequently analysed the functional and phenotypic properties of breast cancer cells transduced with our designed ATFs. As ectopic expression of *maspin* cDNA in breast cancer cells leads to enhancement of apoptosis and decreased cell motility and invasion (Lockett *et al*., 2006), we then studied if the designed ATFs also induced similar phenotypic alterations. To study quantitatively the fraction of apoptotic cells, we performed Annexin-V flow cytometry analysis of cells transduced with our designed ATFs, control cells (transduced with empty retroviral vectors which express GFP) and un-transduced cells. As shown in Figure 3a, ATF-126 and ATF-97 were both able to induce apoptosis in the MDA-MB-468 cell line (55 and 25%, respectively). As expected from its low activity in regulating *maspin*, ATF-452 had no significant induction of apoptosis in this cell line, relative to control (11.8%). In the MDA-MB-231 cell line, only ATF-126 was able to strongly induce apoptosis (70%), whereas ATF-97 and ATF-452 had no significant effect (12 and 24%, respectively) (Figure 3c). Consistent with the capability of ATF-126, ATF-97 (in MDA-MB-468) and ATF-457 (in MDA-MB-231) to induce apoptosis in breast cancer cell lines, we found that these ATFs were able to decrease breast tumor cell proliferation relative to controls (Figure 3a and c, right plots). The differential effect of the ATFs in inducing apoptosis in the two breast cell lines is consistent with the levels of maspin upregulation (Figure 2).

To study if the designed ATFs were able to knock down breast cancer cell invasion *in vitro*, we performed Matrigel invasion assays. As expected from their capability to upregulate *maspin* expression, ATF-97, ATF-126 and ATF-452 were able to knock down cell invasion in the MDA-MB-468 background (37.5, 66.4 and 27.6%, respectively, relative to controls; Figure 3b). Similarly, in the highly invasive MDA-MB-231 cells line, the three ATFs were able to knock down cell invasion (31.5, 77.4 and 44.7%, respectively, relative to controls) (Figure 3d).

To study if ATF-126 was also able to reduce tumor growth *in vivo*, we transduced the aggressive MDA-MB-231-luc cell line (which stably expresses the reporter luciferase) with ATF-126 and with an empty retroviral vector (control). A total of 10^6 transduced cells were injected subcutaneously into the flank of immunodeficient mice ($N=6$). Tumor growth in both groups (ATF-transduced and controls) was monitored both by caliper measurements and also by Bioluminiscence Imaging (by non-invasive *in vivo* monitoring of luciferase activity in these mice; Minn *et al*., 2005). As shown in Figure 4, mice injected with control cells all developed primary tumors, whereas none of the mice injected with ATF-126-expressing cells generated tumors. These data demonstrated that ATF-126 was able to suppress xenograft growth of the MDA-MB-231 cell line in SCID mice.

In summary, our data demonstrate that ATFs can be used to specifically re-activate tumor suppressors that are silenced in metastatic breast tumor cell lines. Although additional work needs to be performed to

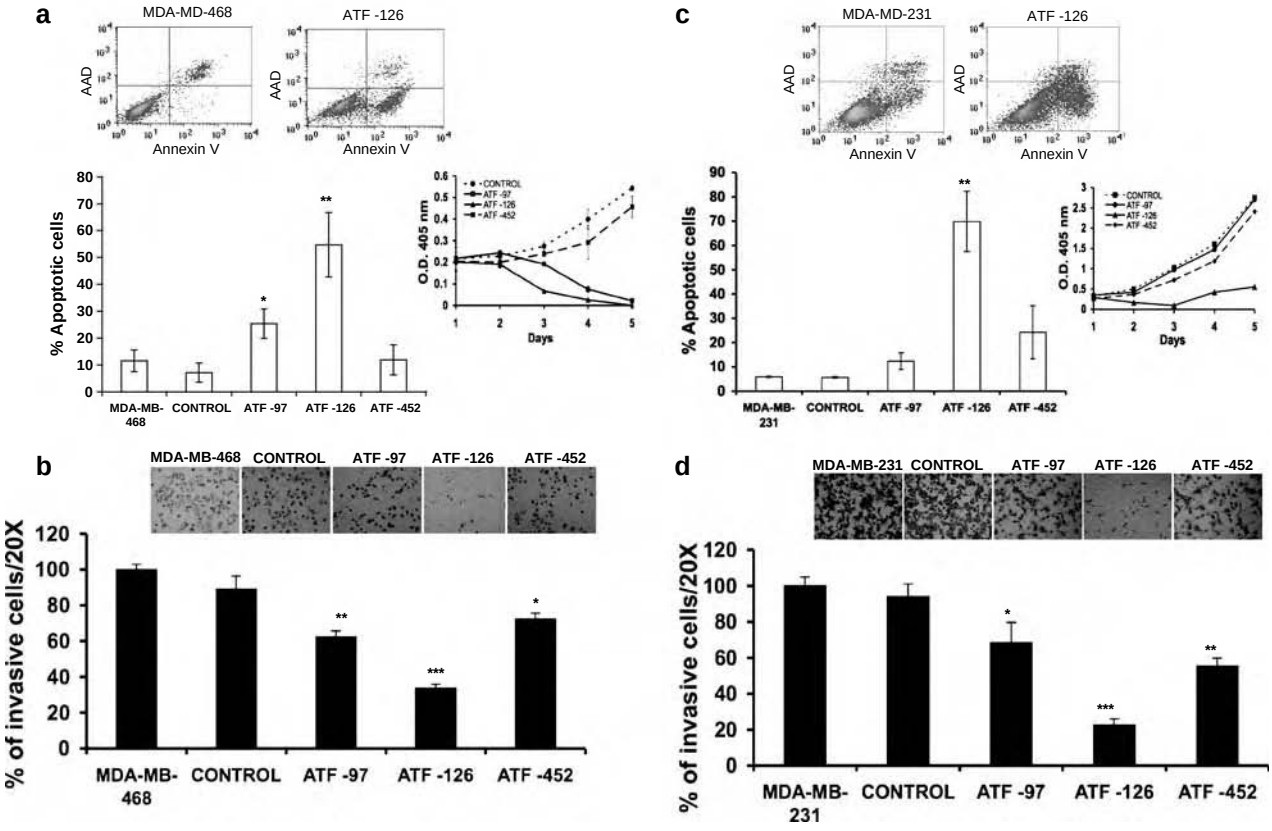


Figure 3 ATFs induce apoptosis and reduce cell invasion in MDA-MB-468 and MDA-MB-231 breast cancer cell lines. ATF transduction of MDA-MB-468 (**a** and **b**) and MDA-MB-231 (**c** and **d**) were performed as described above. (**a**–**c**) Early apoptosis (left plot) was quantitatively analysed in non-transduced cells, control cells and ATF-transduced cells using an Annexin-V staining kit, following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Apoptosis was measured 120 h post-transduction. A positive control was induced with 10 μM camptothecin (Sigma, St Louis, MO, USA) for 24 h prior analysis to setup the flow cytometry settings. The % of apoptosis was measured by flow cytometry using a fluorescence-activated cell sorter Calibur cytometers and Facstation software (BD, Franklin Lakes, NJ, USA). Data represent an average of three different experiments ($*P<0.05$, $**P=0.01$, $***P=0.001$, as determined by Student's *t*-test). The top panel shows a representative flow cytometry result indicating the positive apoptotic population (Annexin-V+ AAD−). Proliferation assays (right plots) were performed using survival assay kits (XTT, Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions. (**b**–**d**) ATF-126 knock down cell invasion when transduced in breast cancer cell lines. Invasion assays were performed using 24-well format Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA) 120 h post-transduction (Supplementary methods). Data represent an average of three different experiments ($*P<0.05$, $**P=0.01$, $***P=0.001$, as determined by Student's *t*-test).

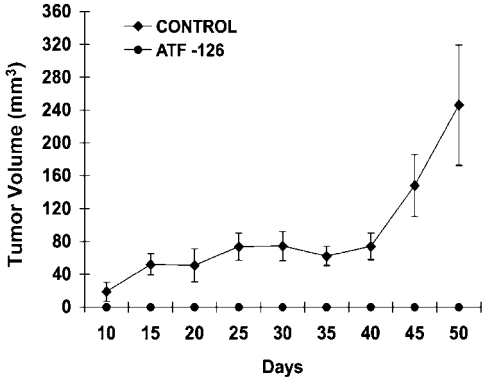


Figure 4 ATF-126 inhibits the growth of the human breast cancer cell line MDA-MB-231 stably expressing a luciferase reporter (Luc) *in vivo*. Growth curve of MDA-MB-231 (Luc) cells transduced with ATF-126 and control (cells transduced with an empty retroviral vector). Control tumor volume gain was 240 mm^3 at day 50. However, no tumor growth was detected in ATF-126-injected mice. Tumor volume = length × $width^2/2$.

evaluate how this ATF exerts its mechanism of action (e.g., if the ATF alters methylation, acetylation status and chromatin accessibility), our work suggests that ATFs could be used in cancer therapeutics as novel strategic intervention to downregulate tumor growth. Given the clinical relevance of *maspin* expression in a variety of epithelial tumors (including breast, prostate, lung and colon), our work describes a novel approach to target multiple human neoplasias.

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Reprogramming epigenetic silencing: artificial transcription factors synergize with chromatin remodeling drugs to reactivate the tumor suppressor *mammary serine protease inhibitor*

Adriana S. Beltran,[1] Xueguang Sun,[2] Paul M. Lizardi,[2] and Pilar Blancafort[1]

[1]Department of Pharmacology and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and [2]Department of Biochemistry and Biophysics, Yale University Medical Center, New Haven, Connecticut

Abstract

Mammary serine protease inhibitor **(*maspin*) is an important tumor suppressor gene whose expression is associated not only with tumor growth inhibition but also with decreased angiogenesis and metastasis. *Maspin* expression is down-regulated in metastatic tumors by epigenetic mechanisms, including aberrant promoter hypermethylation. We have constructed artificial transcription factors (ATFs) as novel therapeutic effectors able to bind 18-bp sites in the *maspin* promoter and reactivate *maspin* expression in cell lines that harbor an epigenetically silenced promoter. In this article, we have investigated the influence of epigenetic modifications on ATF-mediated regulation of *maspin* by challenging MDA-MB-231 breast cancer cells, comprising a methylated *maspin* promoter, with different doses of ATFs and chromatin remodeling drugs: the methyltransferase inhibitor 5-aza-2′-deoxycytidine and the histone deacetylase inhibitor suberoylanilide hydroxamic acid. We found that the ATFs synergized with both inhibitors in reactivating endogenous *maspin* expression. The strongest synergy was observed with the triple treatment ATF-126 + 5-aza-2′-deoxycytidine + suberoylanilide hydroxamic acid, in which the tumor suppressor was reactivated by 600-fold. Furthermore, this combination inhibited tumor cell proliferation by 95%. Our data suggest that ATFs enhance the efficiency of chromatin remodeling drugs in reactivating silenced tumor suppressors. Our results document the power of a novel therapeutic approach that combines both epigenetic and genetic (sequence-specific ATFs) strategies to reactivate specifically silenced regions of the genome and reprogram cellular phenotypes. [Mol Cancer Ther 2008;7(5):1080–90]**

Introduction

Tumor suppressor genes play an essential role in controlling unscheduled cell proliferation and they act as gatekeepers that block neoplastic processes in tissues. Due to the pivotal role of tumor suppressor gene inactivation during tumor progression, these genes are primary targets in cancer therapeutics. Inactivation can occur via a variety of mechanisms, such as point mutations, deletions, and epigenetic modifications (1–3). Epigenetic modifications, such as DNA and histone methylation and histone deacetylation, result in a compact chromatin configuration that silences entire DNA regions (1–6). At the promoter level, this compact chromatin topology restricts the physical access of the polymerase II complex to regulatory sequence domains, resulting in inhibition of tumor suppressor transcription (7–9). Unlike genetic alterations, which irreversibly inactivate tumor suppression expression, epigenetic modifications are potentially reversible (9–11).

The reversible nature of epigenetic silencing offers a unique opportunity for therapeutic intervention by reactivating endogenous tumor suppressor genes. Several chromatin remodeling drugs have been developed to release the repressed state of tumor suppressor genes. These drugs act by inhibiting DNA methyltransferases or histone deacetylases (HDAC), resulting in increased promoter accessibility and enhanced tumor suppressor gene transcription (12–14). To date, the most widely used chromatin remodeling drug is the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-2′-dC), recently approved for therapeutic treatment (15). Several methyltransferase inhibitors [such as 5-aza-dC and MG98 (16, 17)] and HDAC inhibitors [such as suberoylanilide hydroxamic acid (SAHA; ref. 18), valproic acid (19), and pivaloyloxymethyl butyrate (20, 21)] are presently in phase I and II clinical trials. The small-molecule inhibitors 5-aza-2′-dC and SAHA have been used to reactivate tumor suppressor genes aberrantly methylated in aggressive tumor cells, such as *desmocollin 3* (22), *gelsolin* (23), and *mammary serine protease inhibitor* (*maspin*; refs. 13, 22). Moreover, several reports have shown that methyltransferase and HDAC

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Requests for reprints: Pilar Blancafort, Department of Pharmacology and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365. Phone: 919-966-1614. E-mail: pilar_blancafort@med.unc.edu

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inhibitors are able to synergize to reactivate tumor suppressor expression (24, 25). Nevertheless, potential limitations for the use of these drugs in cancer patients include their toxicity, lack of target specificity, and development of acquired drug resistance (6, 26). Thus, there is a need for the development of novel strategies to increase the targeted efficiency and specificity of current anticancer drugs.

Our laboratory has recently applied a new strategy to specifically reactivate tumor suppressor genes silenced by epigenetic mechanisms in aggressive tumors (27). We have targeted the tumor suppressor gene *maspin* using three rationally designed artificial transcription factors (ATFs). These ATFs comprise six sequence-specific zinc finger (ZF) domains, designed to recognize unique 18-bp sites in the *maspin* promoter. The ZFs were linked to the VP64 activator domain, which mediates promoter up-regulation. We found that the capability of the ATFs to up-regulate *maspin* depended on the cell line analyzed, indicating that the structure of the chromatin can influence ATF-mediated transactivation of *maspin*. In the aggressive MDA-MB-231 breast cancer cell line, which comprises a methylated and silenced *maspin* promoter, only one ATF (ATF-126) was able to partially reactivate the endogenous *maspin*. We hypothesized that the structure of the chromatin (which is found in a more compact configuration in methylated promoters) could act as a partial blockade and restrict ATF-mediated transactivation of *maspin*. In this article, we have investigated the influence of chromatin structure at the *maspin* promoter in the context of artificial ATF regulation by challenging MDA-MB-231 cells expressing ATF-126 with different doses of 5-aza-2′-dC and SAHA. We found that ATF synergized with both inhibitors to reactivate *maspin* expression. The strongest synergy was observed with the triple treatment ATF-126 + 5-aza-2′-dC + SAHA, in which the tumor suppressor was reactivated by 600-fold. Furthermore, this combination inhibited breast tumor cell proliferation by 95%. Our data suggest that ATFs amplify the response of chromatin remodeling drugs in reactivating silenced tumor suppressors. Thus, combinations of low concentrations of chromatin remodeling drugs and sequence-specific ATFs are efficient in reactivating silenced regions of the genome and effectively reprogram cellular phenotypes. This could represent a powerful therapeutic strategy to target a variety of neoplasias through specific reactivation of tumor suppressor genes.

Materials and Methods

Cell Lines

MDA-MB-231 breast carcinoma, MDA-MB-468, MCF-12A, and 293TGagPol cell lines were obtained from the American Type Culture Collection.

Sodium Bisulfite Genomic Sequencing of the Maspin Promoter

Genomic DNA (1.5 μg) was modified with sodium bisulfite using EZ DNA Methylation-Gold kit (Zymo Research). The *maspin* promoter was amplified from the bisulfite-modified DNA by PCR using primers specific to the bisulfite-modified sequence of the maspin promoter: 5′-TAGGATTTTAAAAAGAAATTTTTTG-3′ (forward primer) and 5′-CCCACCTTACTTACCTAAAATCACA-3′ (reverse primer). The PCR products were cloned and 10 positive recombinants were sequenced. The methylation status of individual CpG sites was determined by comparison of the sequence obtained with the known maspin sequence.

ATF Retroviral Transduction

The retroviral vector pMX-6ZFs-VP64-IRES-GFP (28) was first cotransfected with a plasmid (pMDG.1) expressing the vesicular stomatitis virus envelope protein into 293TGag-Pol cells to produce retroviral particles. Transfection was done using Lipofectamine as recommended (Invitrogen). The viral supernatant was used to infect the host cell lines, and the infection efficiency was assessed by flow cytometry (FACSCalibur, BD Biosciences) using green fluorescent protein as marker.

Drug Treatments

ATF-transduced cells and control cells (0.25×10^6 untransduced cells, cells transduced with empty retroviral vector, and a control ATF that does not regulate *maspin*) were seeded in 10-cm tissue culture plates. These samples were treated with different concentrations of 5-aza-2′-dC (0, 0.025, 0.05, 0.125, 0.25, 0.625, 1.25, 2.5, 3.75, 6.25, 12.5, 25, 62.5, and 125 μg/mL; Sigma) or SAHA (0, 0.0133, 0.026, 0.066, 0.132, 0.26, 0.4, 0.66, 1.32, 2.6, 3.97, and 5.3 μg/mL; BioVision) or both inhibitors (5-aza-2′-dC and SAHA) during 48 h in a 37°C, 5% CO_2 incubator. Cells were collected, and the RNA was extracted, reverse transcribed, and processed for real-time quantification of *maspin*.

Real-time PCR Expression Assays

ATF-transduced cells and control cells, drug treated or nontreated, were collected, and the RNA was extracted and 2.5 μg were used for reverse transcription. Quantification of *maspin* and VP64 activator domain was obtained by real-time quantitative PCR using fluorescent Taqman assays (Applied Biosystems) as described (27). The primers and probes for the *VP64* activator domain were the following: 5′-AAGCGACGCATTGGATGAC-3′ (forward primer), 5′-GGAACGTCGTACGGGTAGTTAATT3′ (reverse primer), and 5′-6FAM-TCGGCTCCGATGCT-MGBNFQ-3′ (probe). Real-time PCR data were analyzed using the comparative $2^{-\Delta\Delta CT}$ method (SDS 2.1 RQ software, Applied Biosystems) and results were expressed as ''fold change'' in *maspin* RNA expression normalized to *GAPDH* and relative to the vehicle-treated control (29).

Proliferation Assays

Proliferation assays were done measuring cell viability determined by a survival assay (XTT, Roche, according to the manufacturer's instructions). To measure the effect of ATF-126, 5-aza-2′-dC, and SAHA in cell viability, MDA-MB-231 breast cancer cells were transduced with different concentrations of ATF-126 plasmid or/and treated with 5-aza-2′-dC or SAHA (same concentration described earlier). Twenty-four hours after transfection, 3,000 cells per well (5 wells per concentration) were seeded into 96-well format tissue culture plates. Cell viability was

measured using the XTT assay by monitoring the absorbance (405 nm) of the cells at 0 and 72 h after transduction and 48 h of drug treatment.

Experimental Drug Dose-Effect Plots

Dose-effect curves and median-effect plots were generated for each set of the real-time and proliferation data samples using the software package PharmToolsPro (McCary Group; ref. 30). The median-effect dose (Dm_{50}) and the slope (m) were calculated from the median-effect plots and introduced in the isobologram equation for the calculation of the CI (28, 31–33). The CI isobologram equation [$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + (D)_3/(Dx)_3$] was used for data analysis of three-drug combination (31, 32). CI < 1, CI = 1, and CI > 1 indicate synergy, additive effect, and antagonism, respectively.

Statistical Analysis

Real-time PCR and viability experiments were repeated thrice using three independently processed samples. For each sample, we did triplicate acquisitions. Differences between all treatments were analyzed by the ANOVA test with a critical level of significance set up at $P < 0.05$, and significant differences between groups of treatments were analyzed with post hoc Turkish test using the software GraphPad Prism v.5.

Results

ATFs Reactivate *Maspin* in Combination with 5-Aza-2′-dC and SAHA

In a previous report, we have described the construction of three ATFs designed to bind 18-bp sites in the *maspin* proximal promoter (27). The ATFs were constructed by linkage of six sequence-specific ZF domains with the VP64 transactivator domain (Fig. 1A). Each ZF is a compact 30-amino acid domain composed of a recognition α-helix packed with two antiparallel β-strands via the coordination of a zinc ion. The α-helix of each ZF specifically recognizes 3 bp in the DNA or ''recognition triplet.'' The main contact positions are residue +6 of the recognition helix, which interacts with the 5′ nucleotide position of the triplet, residue +3, interacting with the middle base, and position −1, which makes H-bonding contacts with the 3′ nucleotide of the triplet (34).

Our previous results show that the efficiency of *maspin* activation by ATFs depended on the particular cell line analyzed, indicating that the structure of the chromatin may influence the ATF-mediated regulation of the endogenous promoter. To investigate the influence of promoter topology in ATF regulation, we focused our studies on the MDA-MB-231 cell line, an aggressive cell estrogen receptor–negative breast cancer cell line that comprises a methylated and silenced *maspin* promoter (35). First, we verified the methylation status of the *maspin* promoter in the MDA-MB-231 background by doing sodium bisulfate sequencing of the *maspin* proximal promoter and we mapped the ATF-binding sites in this sequence. As shown in Fig. 1B, the ATF-126–binding site contained two methylated cytosines, whereas the ATF-97–binding site comprised one methylated cytosine. In contrast, both ATF-452–binding and the two p53-binding sites in the *maspin* promoter mapped in methylation-free regions. The same methylation pattern was found in other aggressive cancer cell lines comprising a silenced *maspin* promoter (data not shown). The ATF-binding sites were not found mutated or deleted in all 10 genomic clones processed by sequencing. This agrees with previous reports (35–37), which showed that *maspin* gene is not found mutated or deleted in tumor cells, but its promoter is silenced by epigenetic mechanisms. Consistent with this epigenetic silencing, we found that MDA-MB-231 cells have no detectable maspin protein as assessed by Western blotting (Fig. 2C). When retrovirally transduced in MDA-MB-231 cells, only the ATF-126 was able to strongly reactivate the promoter (70-fold relative to controls, in the absence of drugs), whereas ATF-97 and ATF-452 alone had a much weaker activity (27).

To investigate the influence of methylation and chromatin structure on ATF regulation, we challenged ATF-expressing cells with 5-aza-2′-dC and SAHA (Fig. 2). These drugs are known to induce a more relaxed promoter topology, which facilitates the access of transcription factors and DNA polymerase complex (7, 8, 38). 5-Aza-2′-dC causes inhibition of DNA methyltransferase activity. The DNA methyltransferase is bound irreversible to the DNA through the 5-aza-2′-dC residues, which results in a depletion of soluble DNA methyltransferase protein levels. The lack of DNA methyltransferase availability leads to a DNA replication with global demethylation (22, 39–41). SAHA interacts with HDAC enzymes at the catalytic site inhibiting their activity. This process leads to histone acetylation, which opens the chromatin structure, increasing transcriptional activity (42, 43). We hypothesized that remodeling the chromatin in the MDA-MB-231 cell line toward a more open configuration facilitated by 5-aza-2′-dC or/and SAHA enhances the efficiency of ATF regulation of *maspin.* To test this hypothesis, we first retrovirally transduced ATF-97, ATF-126, and ATF-452 into MDA-MB-231 cells. Additionally, cells were transduced with a control empty retroviral vector (control) and with a retroviral vector lacking the ZF domains (pMXVP64SS). These samples were treated with 5-aza-2′-dC (5 μg/mL) or SAHA (0.5 μg/mL) or both inhibitors (5 and 0.5 μg/mL) and processed by real-time PCR for quantification of *maspin* mRNA levels. These concentrations were chosen in the range of the median-effect dose [Dm_{50}, the concentration of inhibitor giving rise to 50% of maximum maspin mRNA up-regulation (31, 32, 44) calculated for these drugs (Fig. 3A–C)]. *Maspin* mRNA levels were calculated as a ''fold change in mRNA expression'' relative to the vehicle-treated MDA-MB-231 cell line as explained in Materials and Methods. Previously, we have found that in the absence of inhibitor only ATF-126 was able to strongly up-regulate *maspin* compared with control cells (cells transduced with an empty retroviral vector), whereas ATF-97 and ATF-452 had a much weaker effect (27). To compare differences between treatments and to evaluate synergisms, we used an ANOVA test with a critical level of significance set up at $P < 0.05$. Significant

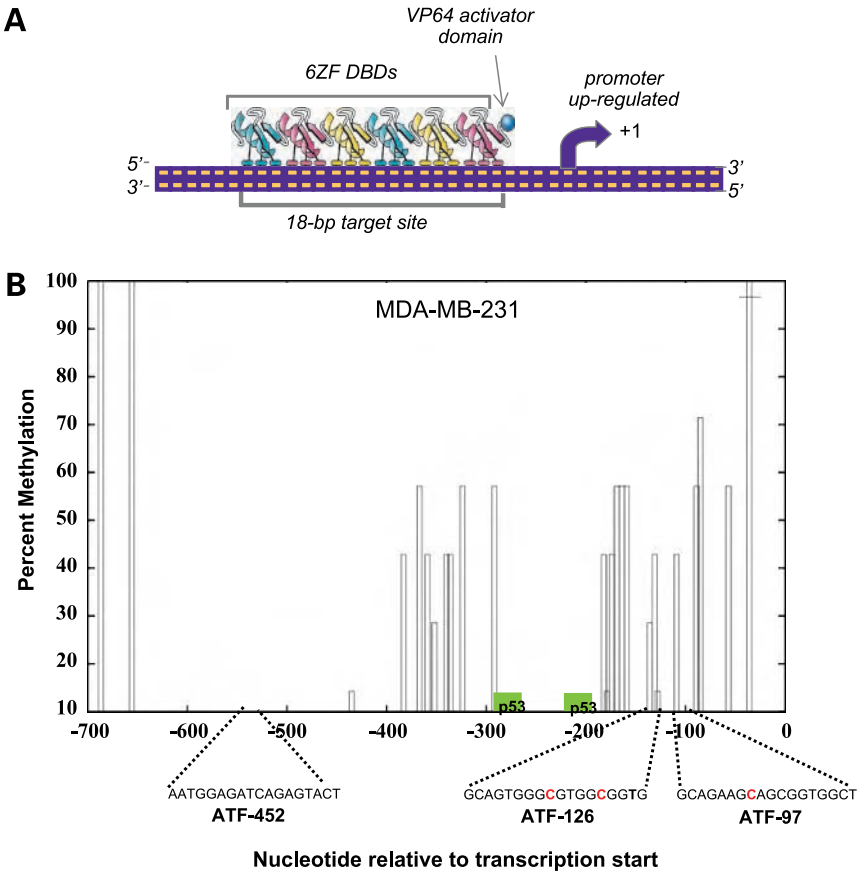


Figure 1. ATFs designed to reactivate the *maspin* promoter. **A,** schematic representation of a 6ZF-ATF. **B,** cytosine methylation status of *maspin* in MDA-MB-231 breast cancer cells. *X axis,* nucleotide position relative to the transcription start site; *Y axis,* percentage of methylation along the *maspin* promoter. The *maspin* proximal promoter region (−495 to +134) was originally reported by Zhang et al. (53). 5-Methylcytosine levels were obtained by sodium bisulfite genomic sequencing of the *maspin* promoter from genomic DNA of untransduced MDA-MB-231 cells. Transcription factor–binding sites were included [p53-binding sites (35) and ATF-binding sites (27)]. Red nucleotides indicate the methylated cytosines in the ATF-binding sites.

differences between groups of treatments were analyzed with post hoc Turkish test.

As shown in Fig. 2A, particular ATFs synergized with chromatin remodeling drugs in reactivating *maspin* expression. ATF-452, which had a poor activity in up-regulating the promoter (3.2-fold), was not able to synergize with 5-aza-2′-dC, SAHA, or both inhibitions in up-regulating *maspin* expression. ATF-97 up-regulated *maspin* by 14-fold and synergized with both inhibitors when used separately: 5-aza-2′-dC (63-fold *maspin* up-regulation) and SAHA (156-fold). However, the triple treatment ATF-126 + 5-aza-2′-dC + SAHA did not significantly further improved regulation. ATF-126–transduced cells up-regulated *maspin* by 70-fold. This ATF synergized with both inhibitors, 5-aza-2′-dC (161-fold) and SAHA (376-fold), in reactivating maspin. In contrast with the other ATFs, the triple treatment ATF-126 + 5-aza-2′-dC + SAHA exhibited synergy, up-regulating *maspin* mRNA levels by 600-fold. This stimulatory effect leads to an 8.26-fold change in *maspin* mRNA expression relative to a breast cancer cell line carrying a nonmethylated promoter (the MDA-MB-468 cell line) and to ~40% of the expression levels observed in nontransformed breast epithelial cell lines, such as MCF-12A (Fig. 2B and C). In contrast with breast cancer cells, nontransformed breast epithelial cells express very high levels of *maspin* (37). Our data suggest that other epigenetic marks, in addition to methylation and histone acetylation, might contribute to maspin silencing in tumor cells.

Because the three ATFs target distinct 18-bp sites along the *maspin* promoter, their particular responses to the inhibitors could reflect local differences in methylation and/or acetylation levels in the chromatin. Overall, these experiments suggested that modifications of the chromatin leading to a more compact promoter topology could partially block or impair ATF regulation, probably by affecting ATF binding.

ATF-126 Synergizes with Low Concentrations of 5-Aza-2′dC and SAHA in Reactivating Maspin Expression

We subsequently focused our studies on ATF-126 because among all the ATFs analyzed it exhibited the strongest response in reactivating *maspin* in combination with chromatin remodeling drugs. High concentration or persistent exposure of tumor cells with chromatin remodeling drugs can potentially result in high toxicity (38, 45). Thus, novel approaches to reactivate tumor suppression expression while minimizing the exposure of tumor cells to the drugs are desired. We next investigated if synergy between the ATF and the chromatin remodeling drugs was maintained when low concentrations of inhibitors (below their Dm_{50}) were used. The Dm_{50} was calculated for each treatment, 5-aza-2′-dC, SAHA, and ATF-126, using dose-effect plots in the MDA-MB-231 breast cancer cell line

(Fig. 3A–D). In these experiments, MDA-MB-231 cells were treated with different concentrations of 5-aza-2′-dC (0.025–125 μg/mL; Fig. 3A) or SAHA (0.07–5.3 μg/mL; Fig. 3B) during 48 h and *maspin* mRNA expression levels were monitored by real-time PCR assays. For ATF-126, cells were transduced with increasing concentrations of ATF-encoded DNA (0–1.3 μg/mL; Fig. 3C), and 72 h after transduction, cells were processed by real-time PCR to detect ATF-126 and *maspin* mRNA levels. The concentration of ATF-126 DNA used in the transfection correlated with ATF-126 mRNA levels detected in MDA-MB-231–transduced cells, as assessed by real-time PCR using ATF-specific primers (Fig. 3D). ATF-126 reactivated *maspin* in a concentration-dependent manner, reaching a maximum effect of 300-fold *maspin* mRNA relative to control cells, whereas 5-aza-2′-dC and SAHA induced a maximum of 13-fold *maspin* up-regulation relative to control cells. The dose-response plots were used to calculate the Dm_{50} for each single treatment. For ATF-126, the Dm_{50} (0.525 μg/mL) was calculated as 50% *maspin* up-regulation at 72 h after transduction. For the inhibitors, the Dm_{50} was calculated as 50% *maspin* up-regulation after 48 h of drug treatment, being 5.55 and 0.75 μg/mL for 5-aza-2′dC and SAHA, respectively (Fig. 3A and B).

To study the synergy between ATF-126 and the chromatin remodeling drugs in reactivating *maspin*, we designed

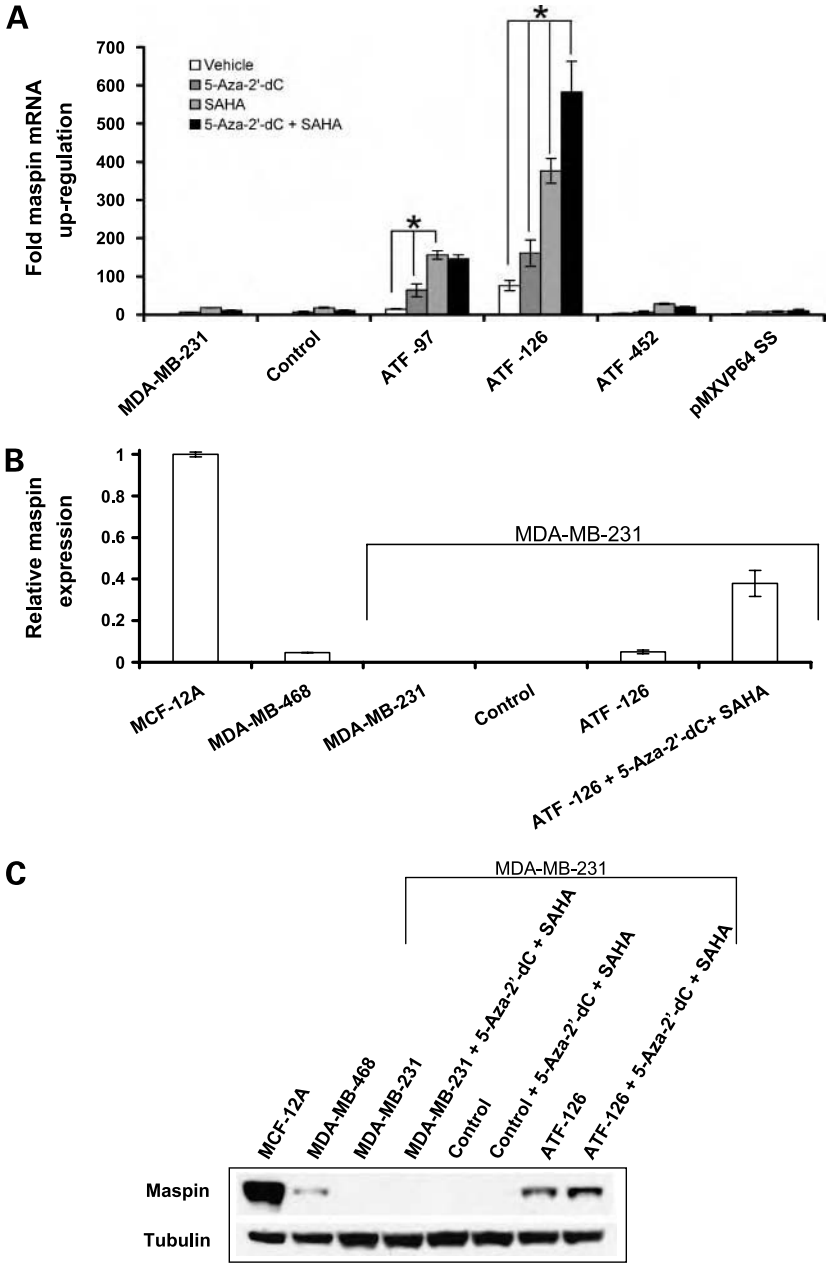
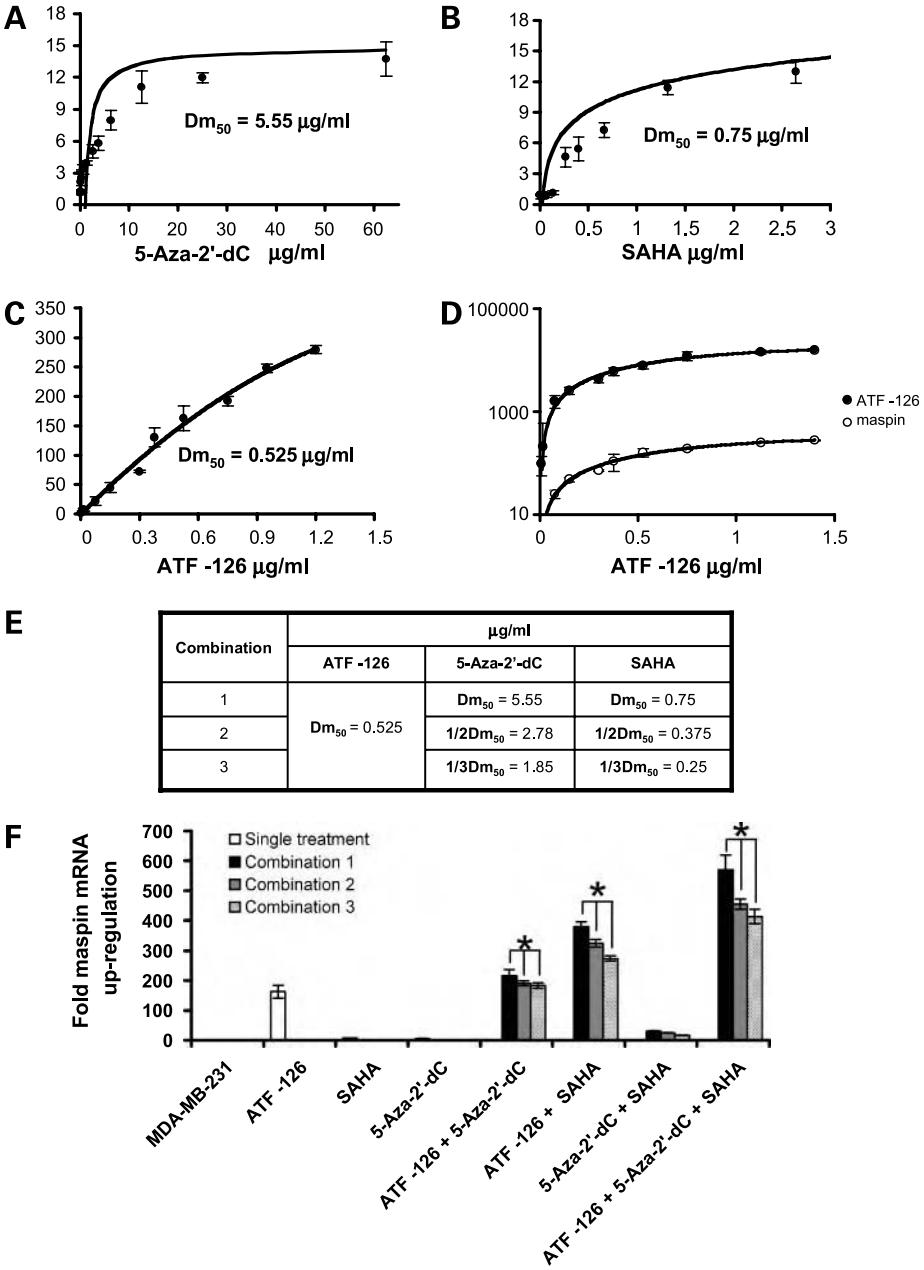


Figure 2. ATFs synergize with chromatin remodeling drugs to reactivate *maspin* expression. **A,** real-time quantification of *maspin* in untransduced MDA-MB-231 cells, cells retrovirally transduced with an empty retroviral vector (control), cells transduced with ATFs, and cells transduced with a control retrovirus lacking the ZF domains (pMXVP64SS). These samples were treated with 5-aza-2′-dC (1.0 μg/mL) or SAHA (0.5 μg/mL) or both inhibitors (same concentrations) using complete cell culture medium for the dilution of the drugs during 48 h in a 37°C, 5% CO_2 incubator. Cells were collected, and the RNA was extracted, reverse transcribed, and processed for real-time *maspin* quantification. Real-time PCR data were analyzed using the comparative $2^{-\Delta\Delta CT}$ method and expressed as fold change in *maspin* mRNA expression normalized to *GAPDH* and relative to the vehicle-treated control (52). Differences between treatments were analyzed using ANOVA test and the post hoc Turkish test; critical level of significance was set up at $P < 0.05$. **B,** real-time expression analysis of *maspin* mRNA levels in the breast cancer cell lines MCF-12A, MDA-MB-468, and MDA-MB-231. MDA-MB-231 cells were transduced with a control empty retroviral vector, with ATF-126, and with ATF-126 in the presence of 5-aza-2′-dC (1.0 μg/mL) and SAHA (0.5 μg/mL). MCF-12A was used as a normalizing control. **C,** Western blot for the detection of maspin in the MCF-12A, MDA-MB-468, and MDA-MB-231 cell lines. MDA-MB-231 cells were transduced with control vector or ATF-126 and treated with a combination of 5-aza-2′-dC/SAHA (0.5 and 1 μg/mL, respectively).



E

Combination	µg/ml		
	ATF -126	5-Aza-2'-dC	SAHA
1	Dm_{50} = 0.525	Dm_{50} = 5.55	Dm_{50} = 0.75
2		$1/2Dm_{50}$ = 2.78	$1/2Dm_{50}$ = 0.375
3		$1/3Dm_{50}$ = 1.85	$1/3Dm_{50}$ = 0.25

Figure 3. ATFs synergize with low concentrations of chromatin remodeling drugs to reactivate *maspin* mRNA expression. **A** and **B,** dose-effect plots assessing changes in *maspin* mRNA levels in cells treated with different concentrations of 5-aza-2′-dC and SAHA. Fold *maspin* mRNA levels were evaluated by real-time PCR using as a normalized control vehicle-treated cells. **C,** dose-effect plots assessing changes in *maspin* mRNA levels in cells transduced with different concentrations of ATF-126. **D,** mRNA expression changes of ATF directly correlate with changes on *maspin* mRNA levels, as evaluated by real-time PCR using primers specific for the ATF and *maspin*, respectively. Changes in mRNA expression of the ATF were generated, varying the amount of ATF-encoded DNA in the retroviral transduction. **E,** concentrations of ATF, 5-aza-2′-dC, and SAHA used in each combination tested. **F,** real-time expression analysis of *maspin* mRNA expression in ATF-transduced cells treated with specific combinations of 5-aza-2′-dC, SAHA, and both inhibitors, as indicated in **E**. MDA-MB-231 cells were transduced with ATF-126 (0.525 µg/mL; the Dm_{50} for *maspin* expression) and treated with three different concentrations (Dm_{50}, $_{1/2}Dm_{50}$, and $_{1/3}Dm_{50}$) of either 5-aza-2′-dC, SAHA, or both compounds. Cells were collected, and the RNA was extracted, reverse transcribed, and processed for real-time quantification of *maspin*. Using fold change in *maspin*, mRNA expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method as described above (52). Differences between treatments were analyzed using ANOVA test and the post hoc Turkish test; critical level of significance was set up at $P < 0.05$.

different drug combination experiments where the ATF-126 was used at its Dm_{50} and the inhibitors were used at their respective Dm_{50} (combination 1), $_{1/2}Dm_{50}$ (combination 2), and $_{1/3}Dm_{50}$ (combination 3; Fig. 3F). As a control, MDA-MB-231 cells were subjected to single treatments (transduced with ATF-126 for 72 h or exposed to 5-aza-2′-dC or SAHA for 48 h) and processed by real-time PCR to evaluate *maspin* mRNA levels. We additionally evaluated *maspin* mRNA levels for double and triple treatments using specific combinations of control cells, ATF-126–transduced cells, 5-aza-2′-dC, and SAHA (Fig. 3E). In all the combinations tested, we found that ATF-126 was able to synergize with 5-aza-2′-dC, SAHA, and both inhibitors to reactivate *maspin*. We found that the most effective combination in activating *maspin* was the triple treatment ATF-126 + 5-aza-2′-dC + SAHA, with a 600-fold *maspin* up-regulation, when all the compounds were combined at their Dm_{50} (drug combination 1). Furthermore, ATF-126 synergized with 5-aza-2′-dC and SAHA in reactivating *maspin* expression by 413-fold even using $_{1/3}Dm_{50}$ of inhibitors (drug combination 3). No statistical difference was observed between combination 2 (when 5-aza-2′-dC and SAHA were used at their $_{1/2}Dm_{50}$) and combination 3 (when 5-aza-2′-dC and SAHA were used at their $_{1/3}Dm_{50}$). Overall, these results indicate that ATF-126

strongly synergized with a combination of methyltransferase and HDAC inhibitors to reactivate *maspin* expression and this synergism was maintained when low concentrations of inhibitors (below their Dm_{50}) were used.

ATF-126 Synergizes with 5-Aza-2′dC and SAHA to Inhibit Tumor Cell Viability

Several reports showed that induction of *maspin* mRNA expression results in inhibition of tumor cell proliferation by enhancement of apoptosis. We next investigated if ATF-126 could also synergize with 5-aza-2′-dC and SAHA in inducing inhibition of tumor cell growth. MDA-MB-231 cells were transduced with a control vector or with ATF-126, and 72 h after transduction, these cells were treated with 5-aza-2′-dC, SAHA, or both inhibitors during 48 h. Tumor cell viability was evaluated using survival assays {2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assays}. The dose-effect plots for ATF-126, 5-aza-2′-dC, and SAHA (Fig. 4A) were used to calculate the inhibitory concentration (IC_{50}, the concentration of ATF-126 or inhibitor giving rise to 50% of inhibition of tumor cell growth at 72 h after transduction or after 48 h of drug treatment). The IC_{50} values were 0.15, 2.056, and 0.942 μg/mL for ATF-126, 5-aza-2′-dC, and SAHA, respectively. To evaluate synergisms, we applied

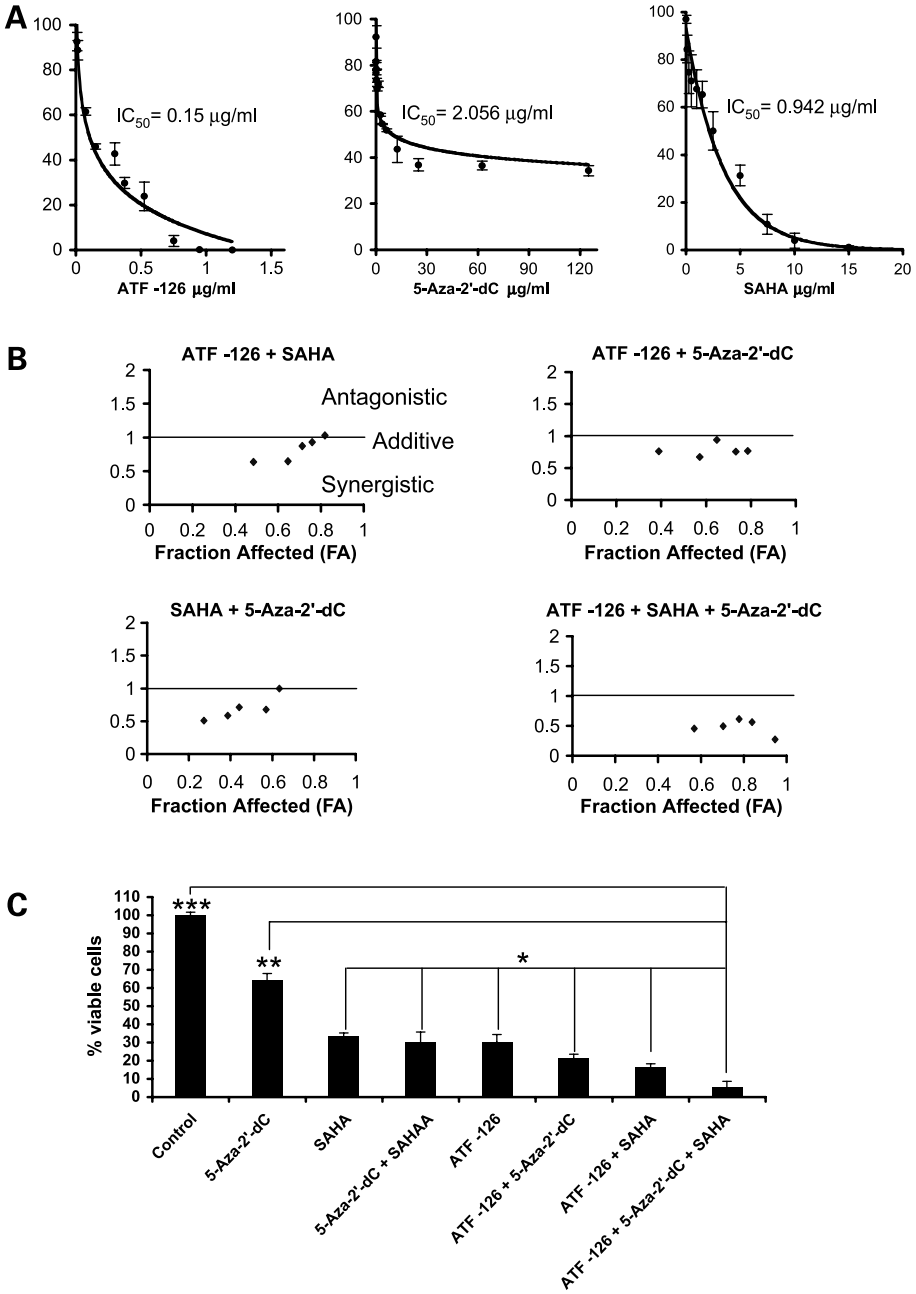


Figure 4. ATF-126 synergizes with 5-aza-2′-dC and SAHA in inhibiting tumor cell viability. **A,** ATF-126, 5-aza-2′-dC, and SAHA induce inhibition of tumor cell viability in a dose-dependent manner. Dose-effect curves for cells transduced with different concentrations of the DNA of ATF-126 or treated with different concentrations of 5-aza-2′-dC and SAHA (Table 1).[3] The effects of the ATFs and the chromatin remodeling drugs in inhibiting tumor cell viability were measured by the ability of metabolic active cells to reduce the tetrazolium salt XTT to orange-colored compounds of formazan. Dose-effect curves and median-effect plots were generated for each set of samples using the software package PharmToolsPro (28). **B,** CI for cells transduced with ATF-126 and treated with 5-aza-2′-dC (ATF-126 + 5-aza-2′-dC), SAHA (ATF-126 + SAHA), and both inhibitors (ATF-126 + 5-aza-2′-dC + SAHA). Nontransduced cells were treated with both inhibitors (5-aza-2′-dC + SAHA). CI was calculated from the median-effect plots (31) to measure the synergistic action between ATF-126, 5-aza-2′-dC, and SAHA in the MDA-MB-231 breast cancer cell line. CI < 1 defines a synergistic interaction, and CI > 1 defines an antagonistic drug interaction. The straight line at CI = 1 represents additive effects. **C,** inhibition of tumor cell viability on ATF-126 transduction and/or treatment with chromatin remodeling drugs. For single treatments, MDA-MB-231 cells were transduced with ATF-126 (0.525 μg/mL) or treated with 5-aza-2′-dC (3.75 μg/mL) and SAHA (1.32 μg/mL) for 48 h at 37°C and 5% CO_2. The same concentrations were used for the following combinations: 5-aza-2′-dC + SAHA, ATF-126 + 5-aza-2′-dC, ATF-126 + SAHA, and ATF-126 + 5-aza-2′-dC + SAHA. Cell viability was measured using the XTT assay, as described above. The data were analyzed using an ANOVA test and a post hoc Turkish test, as described in Materials and Methods. The asterisks indicate that the triple treatment decreased significantly tumor cell viability compared with all the other treatments tested. *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$.

a standard combinatorial method, which uses the isobologram equation to calculate the combinatorial index (CI). The interaction between drugs is defined as synergistic if CI < 1, antagonistic if CI > 1, and additive if CI = 1 (Fig. 4B). Control-transduced or ATF-126–transduced cells were challenged with different concentrations of 5-aza-2′-dC, SAHA, or both inhibitors, as shown in Table 1. We used concentrations of ATF/5-aza-2′-dC/SAHA in the range of the IC_{50} value, which resulted on 30% to 80% of tumor cell growth inhibition. As shown in Fig. 4B, ATF-126 synergized with 5-aza-2′-dC, SAHA, and both inhibitors (5-aza-2′-dC + SAHA) for the majority of the drug combinations tested. The synergistic effect for the double treatments ATF-126 + 5-aza-2′-dC, ATF-126 + SAHA, and 5-aza-2′-dC + SAHA was higher when low concentrations were used in each combination. For the triple treatment ATF-126 + 5-aza-2′-dC + SAHA, we observed a synergistic effect with all the combinations tested. The lowest (most synergistic) CI was achieved by the combination with the highest dose of ATF-126 and inhibitors, which results in 95% of inhibition of tumor cell viability compared with vehicle-treated control cells (Fig. 4C; doses are indicated in Table 1).

As shown in Fig. 4C, the triple treatment ATF-126 + 5-aza-2′-dC + SAHA was significantly the most efficient in decreasing tumor cell viability compared with all the other treatments. The double combinations ATF-126 + SAHA and ATF-126 + 5-aza-2′-dC reduced significantly cell viability compared with all the single treatments (ATF-126, 5-aza-2′-dC, and SAHA and vehicle; $P = 0.05$). However, our data in Fig. 4C did not reveal statistical differences between the following treatments: SAHA, 5-aza-2′-dC + SAHA, and ATF-126. The apparent discrepancy between the RNA and the viability data could be explained by the fact that these experiments measure different outcomes. Unlike real-time, which specifically measures *maspin* mRNA levels, cell viability is a complex phenotype involving many different gene products, including tumor suppressors such as *maspin*. The higher effect of SAHA and 5-aza-2′-dC observed in viability assays might suggest that these compounds reactivate many tumor suppressor genes, not just *maspin* (46, 47). Although off-target effects are possible with ATFs, these proteins have been engineered to reactivate specifically *maspin* and are not expected to regulate other tumor suppressor genes. We are presently investigating putative off-target effects of ATF-126.

To verify that the effect of the ATFs and inhibitors was specific for tumor cells and not normal epithelial cells, we did the same viability assays in a nontransformed breast epithelial cell line, the MCF-12A. In contrast with the MDA-MB-231 cell line, none of the combinations of ATFs and inhibitor was able to significantly up-regulate *maspin* expression nor decrease cell viability as assessed by the ANOVA and post hoc Turkish tests (Supplementary Fig. S1).[3]

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics (http://mct.aacrjournals.org/).

Table 1. CI values for single, double, and triple combinations of ATF-126, 5-aza-2′-dC, and SAHA

ATF/Drug (μg/mL)			fa	CI
ATF-126	SAHA	5-aza-2′-dC		
0.075	0.13215		0.484	0.633
0.150	0.264		0.645	0.647
0.300	0.396		0.713	0.874
0.375	0.661		0.758	0.930
0.525	1.322		0.819	1.028
0.075		0.250	0.389	0.763
0.150		0.625	0.571	0.673
0.300		1.250	0.648	0.939
0.375		2.500	0.733	0.755
0.525		3.750	0.786	0.767
	0.132	0.250	0.272	0.507
	0.264	0.625	0.386	0.583
	0.396	1.250	0.441	0.712
	0.661	2.500	0.570	0.675
	1.322	3.750	0.634	1.000
0.075	0.132	0.250	0.569	0.456
0.150	0.264	0.625	0.704	0.497
0.300	0.396	1.250	0.779	0.613
0.375	0.661	2.500	0.839	0.561
0.525	1.322	3.750	0.947	0.275

NOTE: Experimental dose combinations of ATF-126, SAHA, and 5-aza-2′-dC are indicated. MDA-MB-231 cells were transduced with ATF-126 and treated with 5-aza-2′-CC or SAHA or both for 48 h. Cell viability was measured by using an XTT assay.
Abbreviation: fa, fraction of cells affected by the treatment (no viable cells).

Discussion

In this article, we have investigated the influence of promoter structure in the regulation of the tumor suppressor gene *maspin* by ATFs. We have focused our analysis on the highly invasive, metastatic breast cancer cell line MDA-MB-231, which comprises a *maspin* promoter silenced by methylation and transcriptional repression (35), and on ATF-126, the strongest maspin regulator in this cell line (27). We have challenged MDA-MB-231 cells expressing ATF-126 with different doses of the methyltransferase inhibitor 5-aza-2′-dC and the HDAC1 inhibitor SAHA. These drugs interfere with repressive mechanisms, which maintain inaccessible chromatin structure: aberrant cytosine methylation and recruitment of HDAC complexes. Consequently, these inhibitors are able to relax the chromatin, facilitating access to the polymerase II transcriptional machinery (7–9). We hypothesized that disruption of the epigenetic silencing mediated by methyltransferase and HDAC inhibitors coupled to ATFs would result in an enhanced up-regulation of silenced genes. Our work shows that ATFs synergized with chromatin remodeling drugs to reactivate endogenous *maspin* expression. We found that *maspin* reactivation in response to the inhibitors depended on the ATF-binding site analyzed. It could be that, in the endogenous gene, these sites map in regions of the promoter that contain different levels of histone/methylcytosine modifications. It is also possible that

other endogenous factors, such as additional epigenetic marks in the nucleosome, the positioning of the nucleosomes, and CpG-binding proteins, could affect ATF binding and regulation. The strongest synergy was observed with a triple treatment (ATF-126 + 5-aza-2′-dC + SAHA), in which the tumor suppression was reactivated by 600-fold. Consistent with the tumor-suppressive functions of *maspin*, we found that this triple drug combination was also the most effective in inhibiting breast tumor cell proliferation *in vitro*.

A plausible model explaining this synergy is shown in Fig. 5. In a context of a silenced promoter, methylated CpG islands are associated with methyl-binding proteins, methyltransferases, and HDAC, which maintain the promoter in a compact configuration, inaccessible to the transcriptional machinery (4–6). Likewise, it is possible that in the context of a repressed *maspin* promoter the ATF-binding sites are not optimally accessible to the ATFs. In the ATF, the ZF domains are linked to the strong transactivator domain VP64, which recruits the mediator protein and other polymerase II–associated proteins (including chromatin remodeling enzymes and histone acetyltransferases), resulting in a partial *maspin* reactivation. The synergy between the ATF and the chromatin remodeling drugs could be explained by drug-induced enhanced accessibility of the ATFs for their target sites in the *maspin* promoter.

Methyltransferase and HDAC inhibitors interfere with two enzymatic mechanisms of repression: 5-aza-2′-dC inhibits DNA methyltransferase Dnmt1 enzyme (39, 41, 49), whereas SAHA promotes histone acetylation and weakens the histone-DNA interactions (50). Synergy between methyltransferase and HDAC agents in reactivating silenced tumor suppressors has been previously reported by many groups (14, 22, 25, 51, 52). Our results further show that ATF expression highly amplifies the gene reactivation effect of chromatin remodeling drugs with different mechanisms of action. In contrast with chromatin remodeling drugs, which potentially alter many genes in the genome, the ATF is used as a sequence-specific regulator of tumor suppression expression. Our results agree with a report showing that overexpression of the p53 transcription factor in the p53-deficient MDA-MB-231 cell line leads to a synergy with 5-aza-2′-dC in reactivation of the tumor suppressor *maspin* (13). We have found that, like natural transcription factors, ATFs can strongly synergize with both methyltransferase and HDAC inhibitors. Because ATFs can be designed for virtually any sequence in the human genome, the strategy presented in this article can be potentially applied for the reactivation of any epigenetically silenced promoter. Current ATF technology can generate ATF binding designed sequences with high specificity and selectivity in both *in vitro* binding assays and in reporter transactivation assays. Only a subset of ATFs designed against a given target promoter results in successful endogenous regulation (27), indicating that subtle aspects of the architecture of endogenous promoters may be key determinants for ATF-mediated regulation. Chromatin modifications could limit the binding of the ATFs *in vivo*

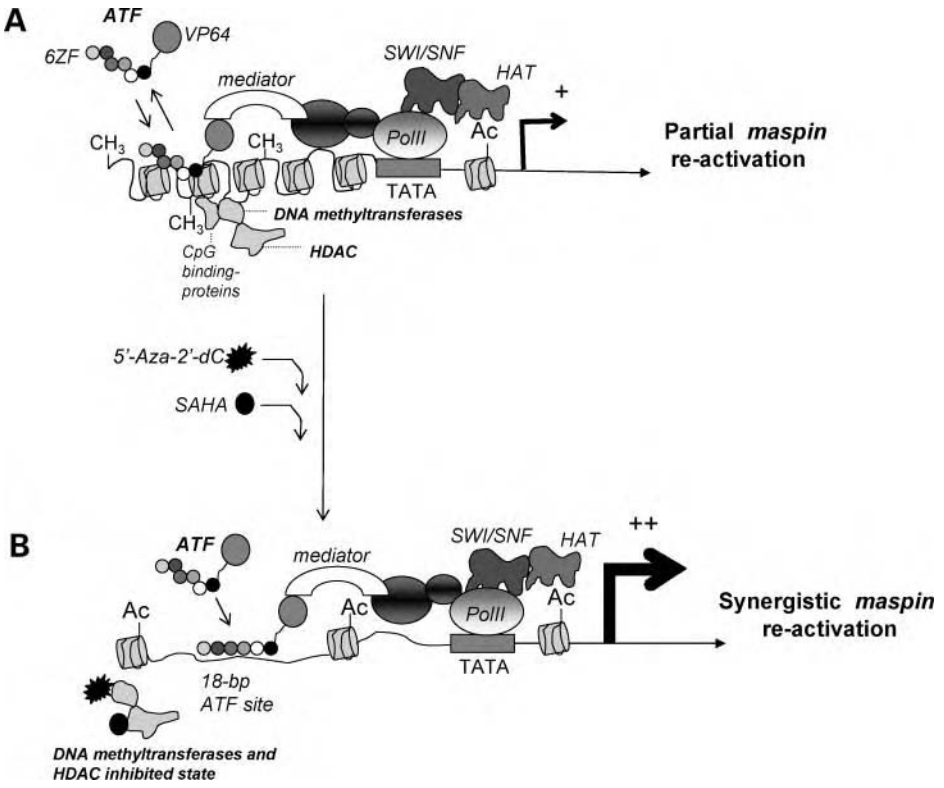


Figure 5. A putative model explaining the synergy between the ATF and the chromatin remodeling drugs in reactivating a methylated *maspin* promoter. **A,** the binding of ATF to the methylated promoter triggers a partial reactivation of the *maspin* gene. **B,** synergistic interaction between the ATFs and chromatin remodeling drugs. On treatment with chromatin remodeling drugs, changes in the chromatin structure facilitate the landing of ATF on the *maspin* promoter, which enhances the *maspin* reactivation.

by restricting ATF target site accessibility. This idea is supported by our observations, which show a gain of ATF-mediated regulation of silenced promoters only in the presence of chromatin remodeling drugs with ATFs having poor or no activity in the absence of remodeling-inducing compounds.

Importantly, we found that strong synergy between ATF/chromatin remodeling drugs was maintained in a concentration range of inhibitors below their IC_{50}. Although more experiments need to be done to evaluate the applicability of our findings to experiments using tumor models *in vivo*, our work shows proof of concept of an exciting strategic approach in therapeutics, which uses ATFs to amplify the apoptotic response of anticancer agents with locus-targeted gene activation while minimizing the exposure/concentration of the drugs.

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